Protein glycation and oxidative stress in pathophysiology of diabetic complications

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Protein glycation and oxidative stress in pathophysiology of diabetic complications

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By

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May26, 2017

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Supervisors' Certificate

This is to certify that the work presented in the dissertation entitled: "*Protein glycation and oxidative stress in pathophysiology of diabetic complications"* submitted by *Jagrati singh*, Roll Number 215BM2257, is a record of original research carried out by him under our supervision and guidance in partial fulfilment of the requirements of the degree of *M.Tech Biotechnology* in *Department of Biotechnology and Medical Engineering*. Neither this dissertation nor any part of it has been submitted earlier for any degree or diploma to any institute or university in India or abroad.

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Declaration of Originality

I, *Jagrati Singh*, Roll Number *215bm2257* hereby declare that this dissertation entitled *Protein glycation and oxidative stress in pathophysiology of diabetic complications* presents my original work carried out as a Masters student of NIT Rourkela and, to the best of my knowledge, contains no material previously published or written by another person, nor any material presented by me for the award of any degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the dissertation. Works of other authors cited in this dissertation have been duly acknowledged under the sections "Reference" or "Bibliography". I have also submitted my original research records to the scrutiny committee for evaluation of my dissertation.

I am fully aware that in case of any non-compliance detected in future, the Senate of NIT Rourkela may withdraw the degree awarded to me on the basis of the present dissertation.

Jagrati Singh
May 23, 2017

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Abstract-

Diabetes mellitus is one of the most prevalent diseases found in the world population. In 2012, according to the WHO report, 1.5 million people died due to diabetic related complications. Type 2 diabetes is a progressive disease. The currently prescribed medication lacks the specificity for targeting particular pathways or molecules involved in the complications. The appropriate understanding of the mechanisms responsible for the diabetes mellitus and its related complications can pave the way to the discovery of the target specific drugs for effective treatment. The current project makes a comprehensive study to get an insight into the probable mechanisms involved and the ways to counteract them. The study specifically centers around *in vitro* induction of BSA glycation and its inhibition, exploration of the effect of extracellular and the intracellular AGEs on the macrophages, protein glycation analysis and inhibition in diabetic serum. The *in vitro* glycation model using BSA confirmed the formation of the both fluorescent and non fluorescent advanced glycation end products (AGEs) in the hyperglycaemic condition. The amount of the AGEs formed increased with the duration of exposure to the hyperglycaemic condition as reflected by increased fluorescent intensity. The oxidative stress induced by hyperglycaemic condition was validated and quantified by NBT assay and H2DCFDA assay. Moreover, the ROS generation in macrophages was increased with addition of extracellular AGEs in the medium. The reduction in the cell viability as a consequence of high ROS production and the presence of the extracellular AGEs was confirmed by MTT assay. During inhibitor study, ascorbic acid exhibited pro-oxidant activity upon adding to the diabetic serum that makes the therapeutic use of ascorbic acid alone debatable unless otherwise verified further. At the same time, GSH and EDTA act as anti-oxidants in macrophages (hence, probably reduced the AGEs formation). In silico docking study was conducted to understand the mechanism of inhibition. The binding affinity of EDTA for BSA was significantly more compared to glucose that didn't reduce much to glycated BSA. This concludes that EDTA can prevent glycation non-competitively and may also halt the progress of glycation of the protein. The results were a step ahead in exploring the novel molecules for inhibiting the protein glycation in T2DM. For a more conclusive finding, a metacentric study involving more number of diabetic patients is needed.

Key words- Advanced glycation end products (AGEs), reactive oxygen species (ROS), EDTA, bovine serum albumin (BSA).

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Chapter 1

INTRODUCTION

Diabetes mellitus is a metabolic disorder in which due to lack of insulin or the body cannot utilize insulin function, leads to high blood glucose level over a prolonged period. This high blood glucose alters the normal body function by altering many pathways. The symptoms of high blood sugar are frequent urination, increased thrust and hunger. The acute and chronic effects due to diabetes include diabetic ketoacidosis, nonketotic hyperosmolar coma and heart disease, stroke, chronic kidney failure, foot ulcers, and damage to the eyes respectively. The types of diabetes mellitus are –

a. Type 1 diabetes, also called as insulin dependent diabetes mellitus (IDDM)-it is characterized by a lack of insulin production. Without daily administration of insulin, type 1 diabetes is rapidly fatal. The development of IDDM is due to several factors such as autoimmune, genetic, and environmental factors.
b. Type 2 diabetes, also called as non insulin dependent diabetes mellitus (NIDDM) results from the body's ineffective use of insulin. About 90% of people with diabetes around the world have type 2 diabetes. The factors responsible for occurrence of NIDDM are older age, obesity, and family history of diabetes, prior history of gestational diabetes, impaired glucose tolerance, physical inactivity, and race.

Regardless of oral treatments or insulin injections almost half of the patients with either type of diabetes develop late complications of neuropathy, retinopathy, nephropathy, and cardiomyopathy. The mechanisms involved in chronic complication of diabetes are under intensive research. The researchers have proposed many theories to establish relation between high glucose level and its long term implications.

Neuropathy is a generalized term frequently used by physicians to indicate defect in sensory perception (attenuated/exaggerated/altered sensation to external stimulus), abnormal visceral regulatory activities and/or partial or complete loss of motor activities of the limbs. The cause of neuropathy is partial (reversible) damage to neurons from primary or secondary causes.

Table 1: Chronic complications associated with diabetic neuropathy-

Health complications from diabetes	Affected region	Occurrence	symptoms
Diabetic retinopathy	eye	Affects 10% people with diabetes	Spots or dark strings floating in your vision (floaters) Blurred vision, Fluctuating vision, and impaired colour vision. Vision loss.
Diabetic neuropathy	Nerves, blood vessels	Affects 50% people with diabetes	Tingling, pain, numbness, or weakness in the feet and hands.
Diabetic nephropathy	kidney	Affects 10-20% people with diabetes	Albumin or protein in the urine, High blood pressure. Ankle and leg swelling, leg cramps. High levels of blood urea nitrogen (BUN)

Neuropathy that arises from sustained hyperglycaemia in diabetic patients (both IDDM and NIDDM) is categorized under Diabetic neuropathy. The medical term for nerve damage, so type of nerve damage due to diabetics is diabetic neuropathy. The nerve most often damaged is of hands and legs.

People with diabetes are prone to develop nerve problem at any time but chances are highest among with uncontrolled glucose level during long term diabetes. The nerves to the feet are the longest in the body and are the ones most often affected by neuropathy.

Nerve damage is due to a combination of factors:

- metabolic factors, such as high blood glucose, long duration of diabetes, abnormal blood fat levels, and low levels of insulin

- neurovascular factors, leading to damage to the blood vessels that carry oxygen and nutrients to nerves
- autoimmune factors that cause inflammation in nerves
- mechanical injury to nerves, such as carpal tunnel syndrome
- inherited traits that increase susceptibility to nerve disease
- lifestyle factors, such as smoking or alcohol use

Chapter 2

LITERATURE REVIEW

Various theories have been proposed to explain patho-physiology of diabetic neuropathy. The most popular among them are the polyol pathway theory, the vascular theory and glycosylation end product theory (11). The major complications of diabetes are peripheral and autonomic neuropathy, micro vascular and macro vascular diseases (11). The frequency of occurrence of peripheral neuropathy is higher compare to autonomic neuropathy as glucose uptake in peripheral nerves is not dependent on insulin (1). The structural and functional abnormalities induced in the diabetic nerve are neuronal ischemia and infractions, endothelial cell hyperplasia, demyelination, axonal degeneration of peripheral nerves, reduced nerve conduction and blood flow (7). The main reason behind demyelination is -due to high glucose myelin is glycated and as a consequence macrophages are activated to secrete protease causing demyelination (7).

The first hypothesis linking hyperglycaemia and nerve dysfunction is polyol (sorbitol) pathway theory. The main enzyme of the pathway involved in pathophysiology is aldose reductase (14).

Table 2: Comparison of pathways involved in normal and pathological condition-

Normal condition-	Pathological condition-
normal blood glucose level	elevated blood glucose
Hexokinase not saturated	Hexokinase saturated(activates aldose reductase)
Most glucose converted to glucose-6 phosphate	Glucose diverted to polyol pathway
No or trace amount of sorbitol detected	Appreciable amount of sorbitol formed
Major pathway-glycolysis	Major pathway-sorbitol pathway
Minor pathway-polyol pathway	Minor pathway-glycolysis

The accumulation of sorbitol leads to tissue toxicity as it is a non charged intracellular osmolytes. The other pathological changes induced are depletion of cytoplasmic osmolytes such as myoinositol, increased aldose reductase activity and interaction with p38 MAP kinase (14, 15).

As a consequence of decreased myoinositol level the level of phosphoinositide is decreased which directly cause reduction in Na/K ATPase activity (9). Activation of aldose reductase depletes its cofactor NADPH. The decreased NADPH level affects many pathways- first there is no formation of reduced glutathione, leads to free radical formation. Secondly NADPH acts as a cofactor for nitric oxide synthase (NOS), thus no NO generation inhibits vascular relaxation causing chronic ischemia (14).

The chronic intracellular hyperglycaemia leads to generation of family of glycation agents known as advanced glycosylation end products (AGE) (2, 7).The various pathways involved in synthesis are (13)-

- Protein glycation via formation of stable intermediates such as amadori products and dicarbonyl intermediates like 3DG (3-deoxyglucosomes).
- Auto oxidative glycation- leads to formation of ketoimine in the presence of transition metal and enediol radical.

Interaction of AGE with its receptor on macrophages increases oxidative stress activating NFκB pathway which leads to formation of various inflammatory molecules. The various in vitro effect of AGE formation observed are-

- Modification of P0 protein with AGE causes demyelination of nerve fibers(1)
- Induced cell death of neuronal and Schwann cells (12)
- Reduced expression of NO synthase thus reduced nerve blood flow.(3)
- The oxidative stress induced by AGE enhances glycation of Na/K ATPase, reducing its activity.(4)

Nitric oxide is nontoxic in biological system as it is rapidly converted to nitrate by reaction with oxy-haemoglobin in RBC(15).In pathological condition both superoxide and NO are synthesized a few cell diameter of each other thus they combine spontaneously to form peroxynitrite.Peroxynitrite modifies various enzymes involved in mitochondrial respiratory chain. It also induces DNA and lipid damage.DNA damage activates PARP (poly-ADP-ribose Polymerase) enzyme .The substrate for PARP is NAD thus lower intracellular concentration of NAD affect pathways for ATP generation.ATP depletion leads to cell death. (16)

To check relation between iNOS mediated peroxynitrite generation studies were carried out on animal models. The mice knocked out for the iNOS gene was protected against chemically induced diabetes. In cardiomyopathy the increased expression of iNOS leads to increased production of nitric oxide and superoxide forming peroxynitrite. The peroxynitrite oxidize

zinc thiolate complex of eNOS, forming uncoupled eNOS which generates more peroxynitrite (17).

As shown in figure 1 – there are two pathways involved in peroxynitrite generation, one with interaction of hydrogen peroxide and hydroxyl free radicals with nitrite to form peroxynitrite and other through increase in iNOS expression during inflammation. The increased expression of iNOS leads to formation of more peroxynitrite.

Peroxynitrite activates Caspase, induces necrosis thus causing cell death (17).The long term hyperglycaemia causes downstream metabolic cascade of polyol pathway hyperactivity, advanced glycation end products (AGE)/receptor of AGE reaction (RAGE), increased reactive oxygen species (ROS). They comprise both endoneurial micro vessels and neural tissues themselves through activation of poly-ADP-ribose polymerase (PARP), alteration of PKC and increase in mitogen activated protein kinase (MAPK), as well as activation of nuclear factor (NF-KB) resulting in functional and structural changes of peripheral neuropathy. Metabolic aberrations in the nerve elicit pro-inflammatory reactions, inducing release of cytokines, suppression of neutrophiles and migration of macrophages and promote development of neuropathy. (18)

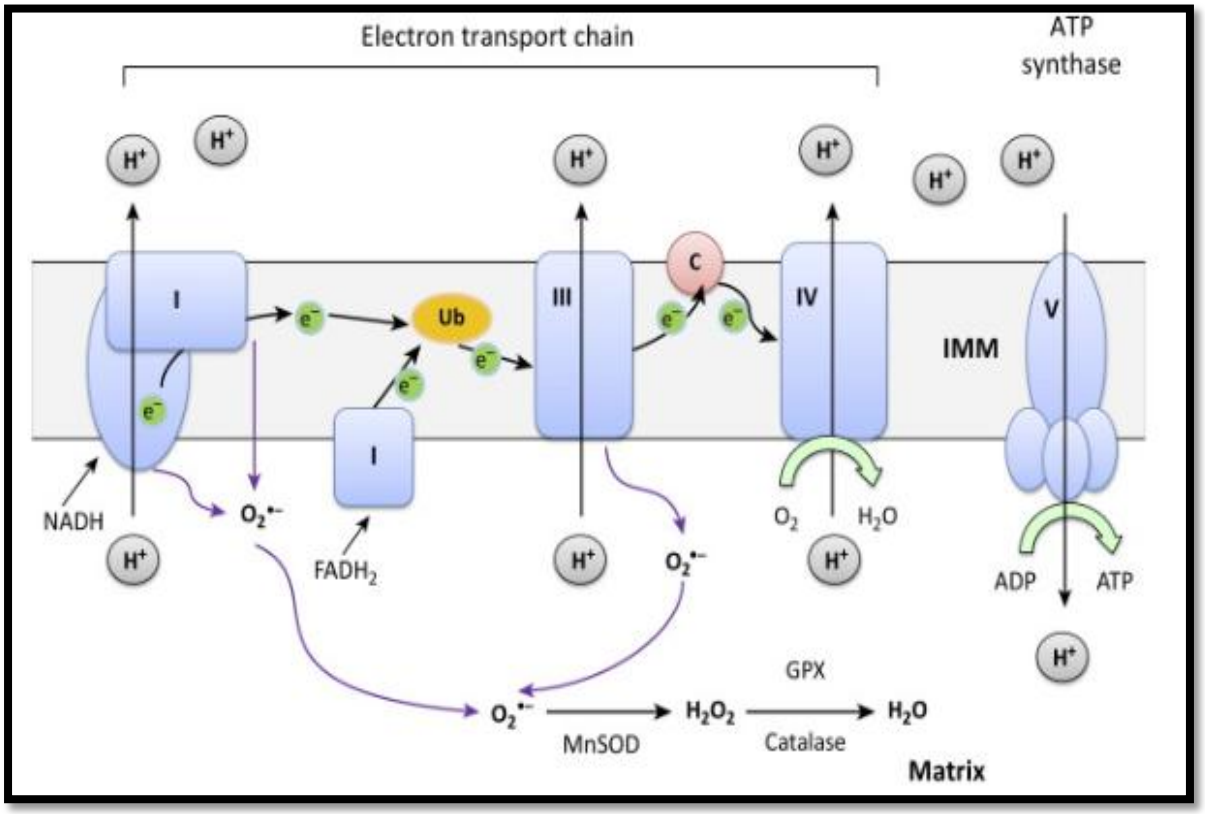

Figure 1 - Electron transport chain (22)

The mitochondria are the major source of superoxide production inside the cell. Under physiological condition the metabolized glucose through citric acid cycle generates electron donor NADH and $FADH_2$ respectively. The NADH donates electron to complex1 and $FADH_2$ to the complex 2(30). The mobile electro carrier coenzyme Q carries these electrons from both the complexes to the final electron acceptor i.e. molecular oxygen (25). Some energy of transported electrons is utilized to pump protons across the mitochondrial membrane. The voltage gradient generated due to proton pumping across the mitochondrial membrane leads to the formation of the ATP. (27)

The high levels of glucose in diabetic conditions generate more electron donors. Due to the electron transport across the four complexes, the established voltage gradient across the mitochondrial membrane increases until the threshold level is reached. After reaching the critical threshold value the complex 3 electron transport is halted. This causes accumulation of the electrons at coenzyme Q (28). At a time coenzyme Q donates single electron to molecular oxygen thus generating superoxides. Thus one of the initial ROS formed inside the cell is superoxide. (30)

The dysfunctional mitochondrion due to redox imbalance inside the cell generates superoxides which have been involved in the development of the diabetic complications. The two major sites have been proposed within the mitochondria, the complex 1 and the Q cycle where electron leakage generates superoxide. Many in vitro studies have postulated that the early disintegration of the mitochondrial membrane potential leads to excessive production of O_2^{-}. (35)

The three damaging pathways are initiated by the hyperglycaemia induced superoxide production. The three damaging pathways are activated due to inhibition of GADPH. All the glycolytic intermediates above the GADPH utilization step are increased in the concentration. This leads to activation of the following pathways (38)-

a. AGE pathway activation- glyceraldehydes 3 phosphate forms the AGE precursor methylglyoxal.
b. Classical PKC pathway activation- the diacylglycerol of the PKC pathway is formed from the glyceraldehydes 3 phosphate.
c. The increased level of the glucose increases flux through polyol pathway

2.1 AGE formation –

The 90% cases reported of diabetes are of type2 diabetes. The type2 diabetic conditions are either due to resistance to the produced insulin or due to less production of the insulin by the

body. The people suffering from diabetes are at high risk of nephropathy, neuropathy, cardiomyopathy, retinopathy and many such complications (35). The microvascular diseases comprise of retinopathy, nephropathy and peripheral neuropathy and the macro vascular complications mainly include cardiomyopathy (36). Three main theories have been proposed to describe pathophysiology of diabetes and its related complications. The three proposed theories are the polyol pathway theory, the micro and macro vascular theory and glycosylation end product theory. The unregulated glucose concentration induces increased influx of glucose through these pathways. All these mechanisms have been interlinked to culminate the redox imbalance inside the body. (38)

Redox imbalance leads to magnificent reactive oxygen species generation by the mitochondrion which in turn again amplifies the functionality of all pathological pathways involved. This viscous cycle of augmentation leads to inevitable consequences of several complications. (40)

2.2 Polyol pathway theory –

This was the first pathway which linked hyperglycaemia with nerve degeneration. Under diabetic conditions

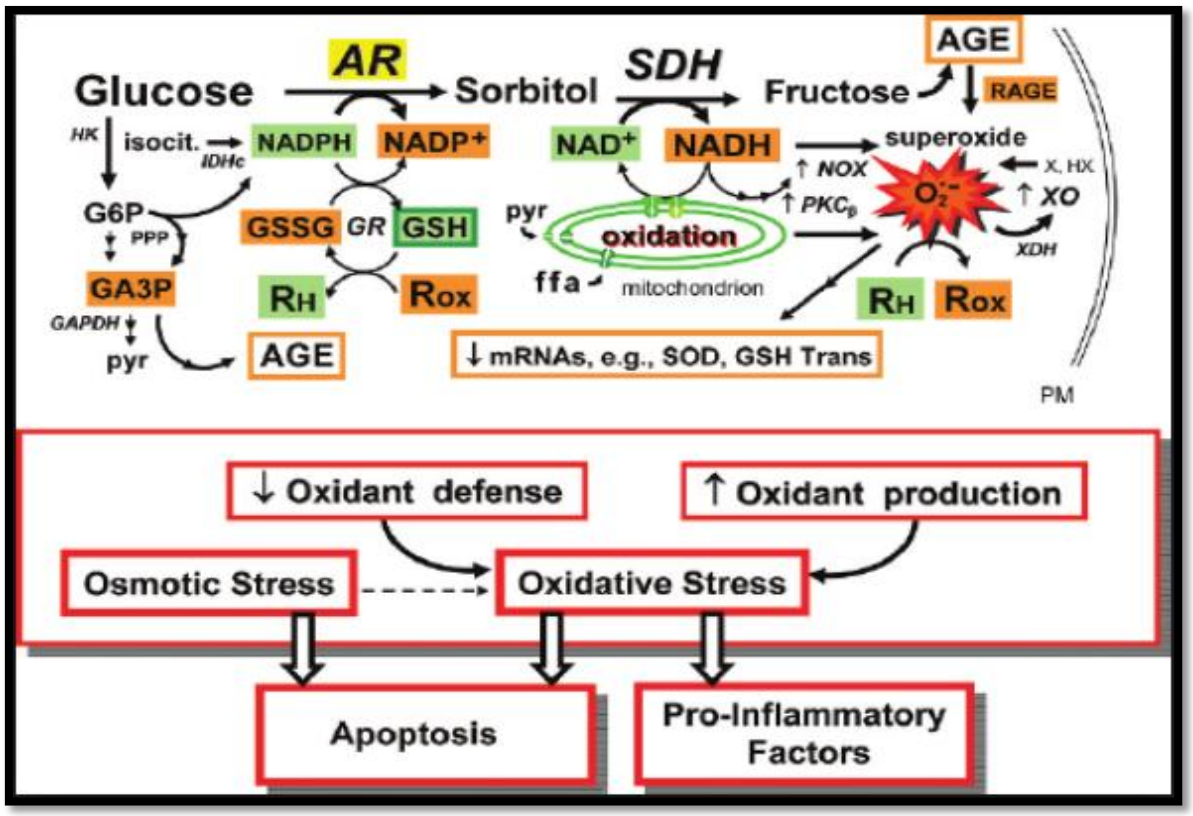

Figure 2- Polyol pathway (14)

Hexokinase is saturated due to high glucose concentration which activates aldose reductase. The activated aldose reductase initiates polyol pathway synthesizing sorbitol (44). The accumulation of the sorbitol inside the tissues causes exhaustion of other osmolytes like myoinositol. The decreased myoinositol level has direct implication of impaired Na/K ATPase activity (48). The concentration of the cofactor NADPH required for the activation of the aldose reductase is also decreased. The decreased level of NADPH leads to reactive oxygen species generation as there is no formation of reduced glutathione (49).

Elevated level of fructose in polyol pathway generates advanced glycation end products. The oxidative stress because of osmotic imbalance and increased oxidant production causes inflammation and programmed cell death.

2.3 Glycosylation end product theory

The non enzymatic glycation of proteins forms fluorescent and non fluorescent advanced glycation end products. The several proteins which are glycated due to hyperglycemic conditions are albumin, fibrinogen, collagen and immunoglobulin. (49)

The in vitro protein glycation has been established by subjecting diverse protein model to hyperglycaemic conditions. The fluorescent intensity peak at 440nm confirms formation of fluorescent AGEs such as pentosidine and cross line with bovine serum albumin.(56) The formation of non fluorescent AGEs like praline, carboxymethyllysine etc. with bovine serum albumin confirmed by measuring m/z ratio of obtained peptide using electron spray ionization mass spectrometry (ESI-MS). The AGE formation leads to activation of several pathological pathways

The one of the major pathway for diabetic complications is formation of advanced glycation end products. The non-enzymatic glycation of the plasma protein such as globulin, fibrinogen and albumin produces several damaging effects like (54)-

- Generation of oxygen free radicals
- Defects in immune system regulation
- Platelet activation and altered drug binding capacity of the glycated plasma proteins

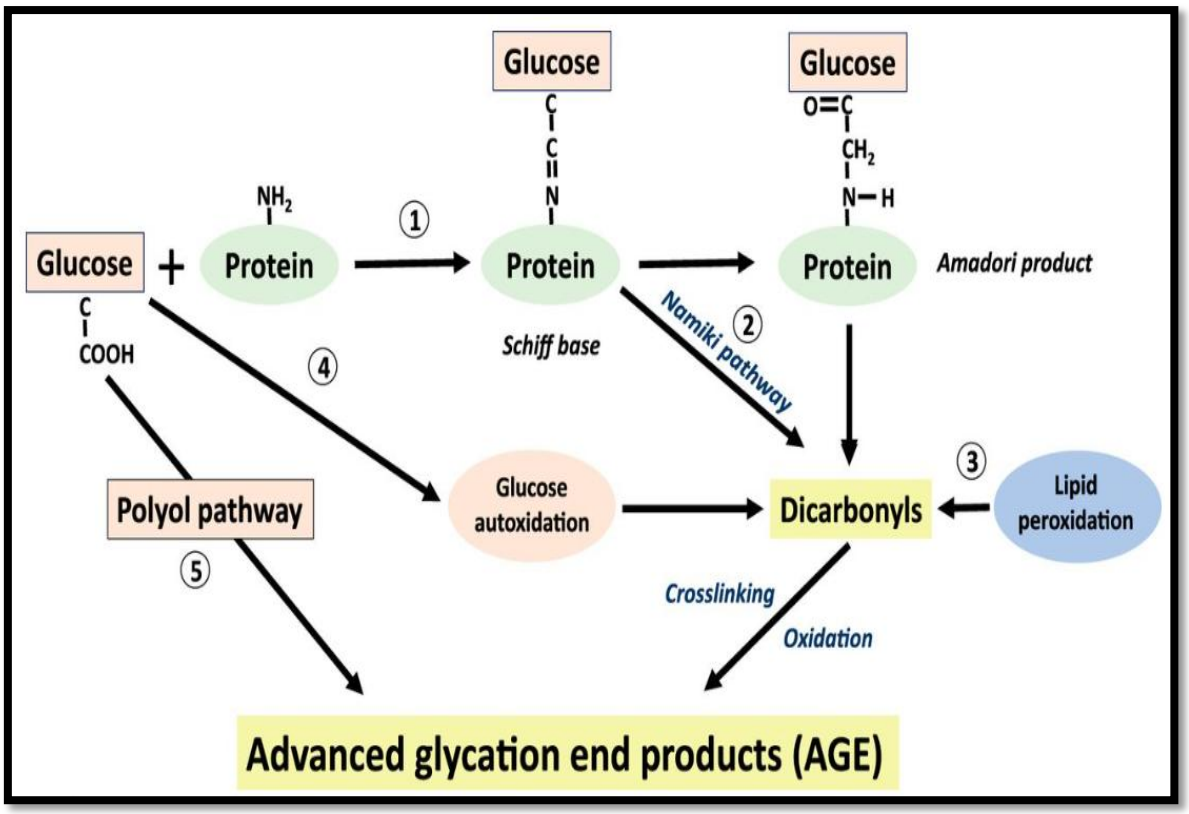

Figure 3- The pathways involved in the formation of advanced glycation end products (59).

The main pathways involved in the formation of the advanced glycation end products are (59)-

- Auto-oxidative pathway- In the presence of free radicals the glucose undergo oxidation to form advanced glycation end products. Thus ROS generation initiates advanced glycation end product formation.
- Amadori rearrangements- The non enzymatic interaction of the amino acid with the carbonyl group of the reducing sugar generates a reversible product known as Schiff base. The Schiff base further undergoes process of cyclization to form more stable amadori products. The amadori products form advanced glycation end product by two ways either oxidative cleavage or non- oxidative cleavage. In oxidative cleavage the intermediates formed undergo process of autoxidation and rearrangements to form carboxymethyl-lysine. The non oxidative cleavage of amadori products form 3-deoxyglucosane , which further reacts with amino acids to form crosslink AGEs such as , pentosidine, or imidazolone.

- Namiki pathway- Alpha dicarbonyl are the main precursor in the AGE formation. In this pathway Schiff base forms glyoxal precursor for AGE formation.

- Wolff pathway- Auto-oxidation of the glucose accelerates the formation of the alpha dicarbonyl precursor which enhances AGE formation.
- Glycolytic pathway- the intermediates of the glycolysis such as glucose-6-phosphate, glyceraldehydes-3-phosphate and dihydroxyacetone phosphate generates alpha dicarbonyl precursor forming advanced glycation end products.
- Lipid peroxidation- The reactive oxygen species causes lipid peroxidation. Lipid peroxidation generates dicarbonyl precursors for the formation of the advanced glycation end products.

The glycation of the human serum albumin alters the structural and functional properties of this plasma protein in many ways (60)-

- Becomes less efficient for transporting long chain fatty acid
- Generation of intracellular reactive species.

The important factors affecting the non-enzymatic glycation of proteins both in vivo and in vitro are (55)-

i. Glucose concentration-the high the glucose concentration the more the formation of advanced glycation end products.
ii. Duration of exposure to the high concentration of the glucose- the longer the exposure time to hyperglycemic condition , high the concentration of AGEs formed.

2.4 Classification of AGEs-

The three main groups in which advanced glycation end products has been classified are based on the structural properties.

Table 2- Types of AGEs formed-

Name of AGEs	Fluorescent-cross linking AGEs	Non-fluorescent cross-linking AGEs	Non-cross-linking AGEs
Examples	Pentosidine crossline	Imidazolium dilysine cross-links Alkylformyl glycosyl pyrrole cross-links Arginine-lysine imidazole cross-links	Pyrraline N-carboxymethyl lysine(CML)

2.5 AGE – RAGE interaction-

The receptors for AGEs are expressed on many cells in vivo for many cellular metabolic activities. The cells expressing these receptors are macrophages, endothelial cells, astrocytes and smooth muscle cells. In diabetic conditions there is both enhanced formation of the advanced glycation end products and also increased expression of the RAGE. (42)

AGE-RAGE interaction increases NFκB expression which transduces pro-inflammatory, and pro-apoptotic signals inside the cell. Formation of NFκB also leads to increased expression of the RAGE on the cell surface. The interaction has also been linked to activation of NADPH oxidase and mitochondrial membrane depolarization thus inducing ROS generation in the cell. (48)

Apart from the RAGE receptor, AGE also interacts with the several other receptors expressed on the surface of the cells. These receptors are lactoferrin, scavenger receptor type1 and 2, CD36, oligosaccharyl trasferase -48, 80 K-H phosphoprotein. (59)

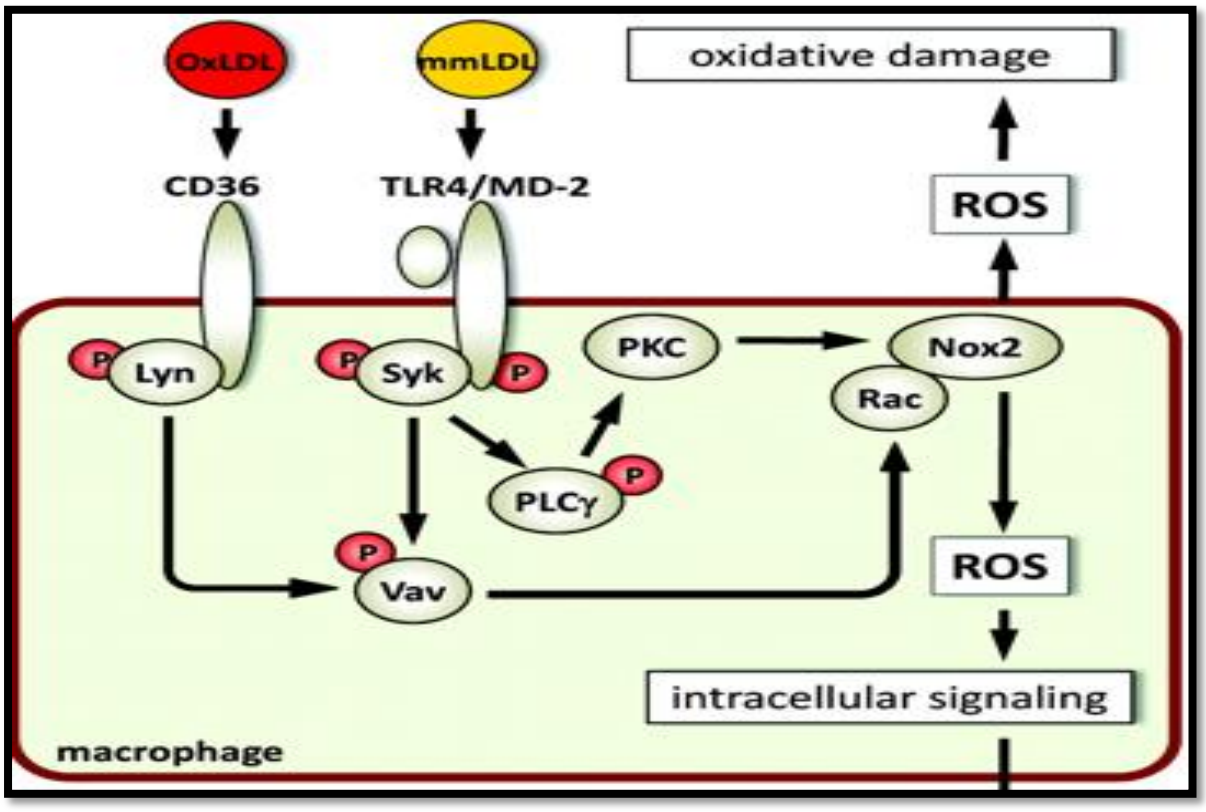

Figure 4- The activation of NADPH oxidase with CD36 cell surface receptor on the macrophages. (58)

The CD36 receptor expressed on the macrophages is a multiligand receptor. One of the interaction pathways of this receptor with oxidized low density lipoprotein in the macrophages activates NADPH oxidase. (54) The activation of the NADPH oxidase leads to

superoxide production inside the cell. The AGE not only leads to ROS generation but also structural changes are induced in the proteins leading to the impaired function. The mitochondrial dysfunction leading to the oxidative stress is as a consequence of the non-oxidative dicarbonyl glycation. The enhanced ROS generation under the hyperglycaemic condition due to advanced glycation end product formation cast towards the activation of the pathways which actuate NADPH oxidase and mitochondrial superoxide generation. The structural changes in the mitochondrial membrane proteins initiates' dicarbonyl intermediate formation actuating intracellular advanced glycation end product formation which causes loss of mitochondrial membrane potential forming ROS.

Thus both intracellular and extracellular advanced glycation end products induce cells to generate more ROS in the hyperglycaemic conditions. (56)

The activation of NADPH oxidase generates superoxide, the enzymatic defence against superoxide is activated, and in this case superoxide dismutase (SOD) reduces superoxide to hydrogen peroxide, which leads to increased expression of eNOS. The nitric oxide synthesized from eNOS combines with the generated superoxides to form peroxynitrite. The oxidation of the essential co-factors for eNOS by the peroxynitrite forms uncoupled e NOS which only generates superoxide without formation of nitric oxide. As a consequence amplification of the intracellular ROS generation occurs inside the cell.

2.6 Role of glutathione –

Glutathione is synthesized in the body form the amino acids L-cysteine, L-glutamic acid, and glycine. It is regarded as the mother of all the anti-oxidants (51). Glutathione relieves cell from the oxidative stress as glutathione the reduced form accepts electrons from the free radicals getting oxidized to form oxidized glutathione (53). The reduced form of the glutathione in the cell is maintained by the cofactor NADPH which donates electron to the oxidized glutathione to replenish the antioxidant glutathione. The alteration in the concentration of the NADPH cofactor in the cell correlates to the oxidative stress as the reduced glutathione is not recycled to react with the superoxides.

2.7 Role of vitamin c-

Vitamin c has both antioxidant and pro-oxidant activity. The concentration of the vitamin c used is the deciding factor whether it acts as anti-oxidant or pro-oxidant. The interaction of the vitamin c with the metal ions generates hydroxyl free radical through the fenton pathway. In chronic inflammation the concentration of the unbound iron increases (55). The free iron is

tremendously toxic to the cells. In the fenton reaction ascorbic acid recycle the ferrous iron forming dehydro ascorbate (42). The ferrous iron reacts with the oxygen generating superoxides. The reaction has been linked to the oxidation of proteins, membrane lipids and also occurs in the presence of the biological reducing agent such as ascorbic acid. The anti-oxidant activity of the vitamin c is determined by the concentration of the free metal ions in the body and the amount of vitamin c used as high concentration usually has pro-oxidant effect. Thus catalytic metal ions such as iron and ascorbic acid cocktail have damaging effect on the cells (58).

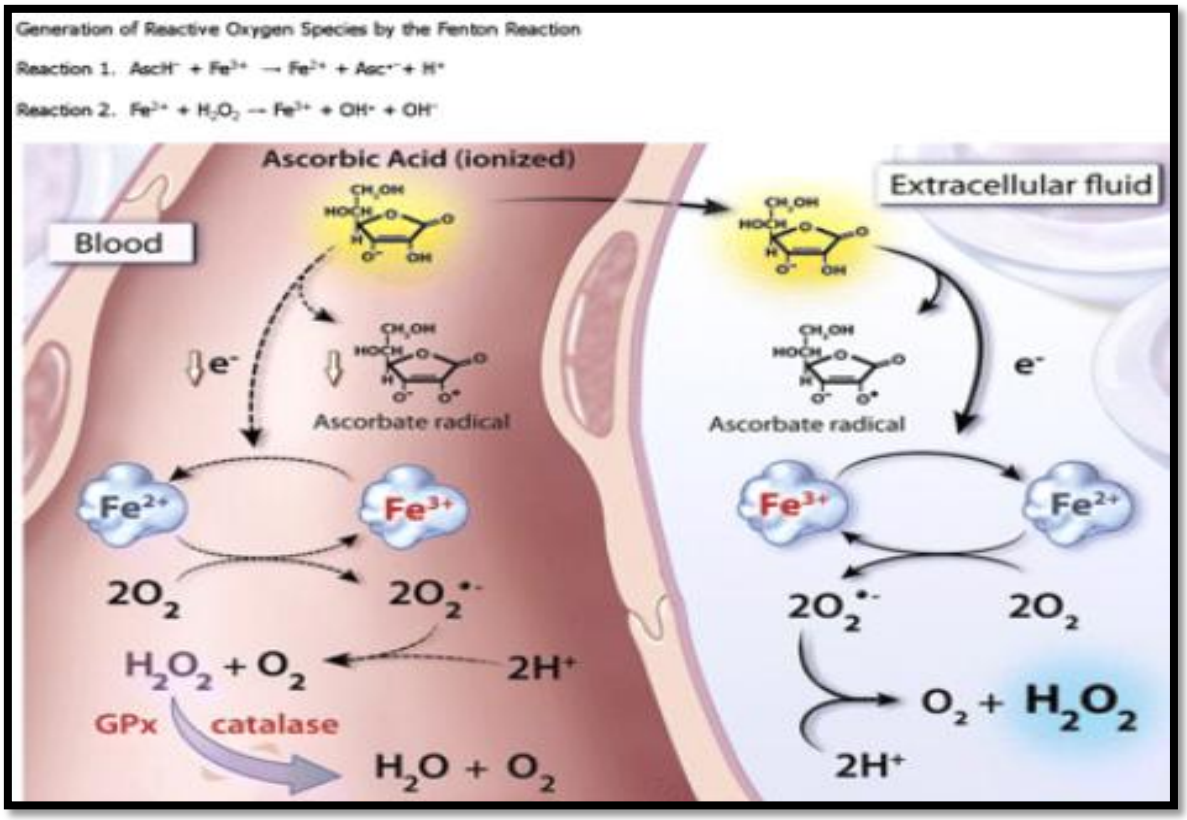

Figure 5- Pro-oxidant activity of the ascorbic acid

2.8 Types of free radicals formed in the body-

The balance between the free radicals generated in the body and the enzymatic and non-enzymatic anti-oxidant systems activity helps in maintaining the normal homeostasis of the body (59). The increase in the free radical formation and the decreased activity of the available anti-oxidant system is responsible for the generation of the oxidative stress. In the physiological conditions the generated superoxide from the complex 3 of the mitochondria are reduced to hydrogen peroxide by superoxide dismutase. In pathological conditions the impaired functionality of complex 1 leads to excessive ROS production from the mitochondria and the subsequent reduction in the activity of the free radical scavengers

enhances the level of ROS in the cell causing oxidation of the proteins, lipids and ultimately cell death (54). The mitochondrion is the main source for the intracellular ROS generation. NADPH oxidase is the source for the extracellular ROS in the phagocytic cells like macrophages. Thus both extracellular and intracellular ROS is responsible for diabetic related complications. (60)

Objectives

In vitro glycation studies with bovine serum albumin (BSA)-

- To prove *in vitro* glycation of bovine serum albumin and to identify the different types of AGEs formed and to establish structural change due to glycation

To investigate effect of high glucose on macrophages -

- Checking viability
- To check ROS generation and the types of ROS produced
- To investigate intracellular AGEs formation

To check AGEs formation in the diabetic serum

To carry out inhibitory studies with the *in vitro* and diabetic serum

Chapter3

MATERIALS AND METHODS-

3.1 Advanced glycation end product formation with Bovine serum albumin -

3.1.1 Materials required – Bovine serum albumin (BSA), phosphate buffer saline, glucose, autoclaved water

3.1.2 procedure-

2mg/ml of BSA used for making 3 solutions with different glucose concentration.BSA, PBS, water, glucose was mixed in the ratio of 2:5:2:1.The glucose concentration added in the range of 1mg/ml and 3mg/ml.The prepared samples were incubated at 37ºc for 1week.The AGE formation was confirmed by measuring fluorescence intensity at excitation and emission wavelength of 370nmand 440 nm respectively.

3.2 Circular dichroism analysis of the glycated BSA-

3.2.1- Materials required- BSA, buffer, autoclaved water, CD instrument

3.2.2 Procedure-

1mg of the bovine serum albumin was dissolved in the 5ml of the 1:1 ratio of the phosphate buffer saline and autoclaved water. Three samples containing BSA, BSA with 1mg/ml of the glucose and BSA with 3mg/ml of the glucose respectively were prepared in the 5ml of the stated solution. After one week of the incubation at 37ºc the samples were scanned in the 190 to 260 nm range for the detection of the secondary structures present in the protein.

3.3Detection of non-fluorescent AGEs using ESI-MS-

3.3.1 Materials required- HPLC Grade water, bovine serum albumin, d-glucose, 45nm filter

3.3.2 Procedure-

The samples were prepared by dissolving 1mg of the protein in 10 ml of the HPLC grade water. As the concentration of the protein used should be less than 50 ppm. The two samples were prepared, one with only BSA and the other with BSA + 3mg/ml of the glucose. The prepared samples were incubated for the week at 37ºc. Before analysis the samples were syringe filtered. The m/z ratios of the peptides were obtained.

3.4 Inhibitory studies for the formed AGEs using fluorometer-

3.4.1 Materials required- BSA, disodium EDTA, glucose, glutathione, vitamin c, PBS, autoclaved water, incubator, fluorometer

3.4.2 Procedure-

The 1.5 mg of the di-sodium EDTA was added to the samples prepared with the BSA and glucose. 2.5 mg of the glutathione and vitamin c each added to the samples with the BSA and glucose. The combination of these inhibitors was also used. After one week of the incubation with inhibitors the sample were analyzed in the emission spectra of 440nm with fluoromax fluorometer.

3.5 Culturing macrophages under high glucose condition-

3.5.1 Materials required- RAW 264.7, DMEM low glucose media, syringe filtered glucose, antibiotic, fetal bovine serum, 6-well plate

3.5.2 Procedure-

Four complete media were prepared containing 2mg/ml; 3mg/ml and 4mg/ml glucose concentration respectively and equimolar concentration of the mannitol.Cells were scrapped and pelleted down to culture in 6 well plates containing 2ml of media with different concentration of glucose. The cultured cells incubated at 37ºc for further analysis.

3.6 NBT assay for measuring superoxide production-

3.6.1Materials required- freshly prepared NBT dye, DMSO, cells seeded in 96 wells plate, 96 well plate spectrophotometer

3.6.2 Procedure-

The cells seeded in the 96 well plates on 8^{th} and 16^{th} day of incubation period were analyzed for superoxide production.5µl of NBT dye added to 50µl media containing cells and incubated at 37ºc for 2hrs.After incubation the complete media was remove and 140µl of DMSO was added. After 5 minutes of incubation absorbance measured at 620nm.

3.7 MTT assay for cell viability-

3.7.1 Materials required- MTT dye, DMSO, cells seeded in 96 wells plate, 96 well plate spectrophotometer

3.7.2 Procedure-

10µl MTT dye was added to 100µl of media containing cells with different glucose concentration at 8^{th} and 16^{th} day of incubation. The samples were incubated for 4 hrs at

37ºc.After incubation the whole media was removed and 100µl of DMSO was added. After 15 minutes of incubation absorbance reading at 595 nm was measured.

3.8 assay for measuring ROS production-

3.8.1 Materials required- freshly prepared H2DCFDA dye, 96 well plates, macrophages, 96 well plate spectrophotometer

3.8.2 procedure-

100µl of media containing cells were poured in 96 well culture plate After 24 hrs incubation media was removed.30µl of dye was added to each well. Cells were incubated for 30 minutes and fluorescent intensity measured at 523nm.

3.9 The in vitro glycation of the human serum albumin-

3.9.1 Materials required- purified human serum albumin, d-glucose, phosphate buffer saline, autoclaved water, and incubator.

3.9.2 Procedure-

The 2mg/ml of the protein concentration was used for the preparation of the samples with the different glucose concentration. The 2mg/ml, 3mg/ml and 4mg/ml of the glucose concentration was dissolved in 1:1 ratio of the PBS and water containing 2mg/ml of the protein in each sample. The prepared samples were incubated at 37ºc for the one week. The formation of the fluorescent AGEs confirmed by the visualization of the sample at excitation of 370nm and emission range of 440-470nm in confocal microscope.

3.10 To visualize the formed AGEs with the confocal microscopy-

3.10.1 Material required- the one week glycated sample of the human serum albumin, fixtures, confocal microscope.

3.10.2 procedure-

The fixtures were prepared. Before the analysis the samples were poured in the fixtures. The fluorescent intensity was measured in the emission range of the 440-470 nm.

3.11 H2DCFDA – dye for the visualization of the ROS-

3.11.1 Material required- formed AGEs with in vitro model, cells cultured in high glucose, H_2DCFDA dye, fluorometer

3.11.2 Procedure-

The 20 µl of the one week incubated human serum albumin with 2mg/ml, 3mg/ml and 4 mg/ml of the glucose concentration respectively was added to the macrophages grown in the 2mg/ml, 3 mg/ml and 4mg/ml of the glucose concentration in the media. After one week of incubation with the advanced glycation end products the cells were scrapped and centrifuged. The obtained pellet was resuspended in the1 ml of the fresh media. The 2*10^5 cells/ml were taken from all the samples and 30µl of the dye was added to the each sample. After 30minutes of the incubation the fluorescent intensity was observed in 523nm emission range.
The cells cultured in 2mg/ml and 4 mg/ml concentration of the glucose were from their 2*10^5 cells/ml were taken and 1.5 mg of the EDTA and 2.5mg of the glutathione was added. After 24 hrs of the incubation in the co_2 incubator, the 30µl of the dye was added to each sample and the fluorescent intensity was measured in the emission range of 523nm.

3.12 Isolation of serum from diabetic blood-

3.12.1 Materials required- serum selection tube containing ficoll, diabetic blood, centrifuge

3.12.2 Procedure-

The diabetic blood was collected without adding anticoagulant in the serum selection tube. The blood was kept at room temperature for half an hour .The collected blood was centrifuged at 1000*g for 10 minutes .Above ficoll layer a distinct layer of serum was collected in sterile tube and stored at 4^oc for further analysis.

3.13 Inhibition of AGE in diabetic serum using EDTA-

3.13.1Materials required- isolated serum, Ethylenediaminetetraacetic acid (EDTA), glucose, autoclaved distilled water, phosphate buffer saline (PBS), fluorometer

3.13.2 Procedure-

1.5 mg/ml EDTA was added to the 5ml solution containing PBS and autoclaved water in 1:1 ratio. The fluorescence intensity was measured to confirm inhibition at excitation wavelength of the 370nm and emission wavelength 0f 440nm.

Chapter4

RESULTS AND DISCUSSIONS

4.1 In vitro advanced glycation end product formation (AGE) formation in bovine serum albumin (BSA)-

The fluorescent advanced glycation end products (AGEs) formation was confirmed by the emission peak at the 440 nm.

In the Millard reaction the glucose carbohydrate group reacts with the amine group of the protein forming Schiff base which rearranges to form amadori products, these unstable amadori products leads to formation of irreversible AGE (6).

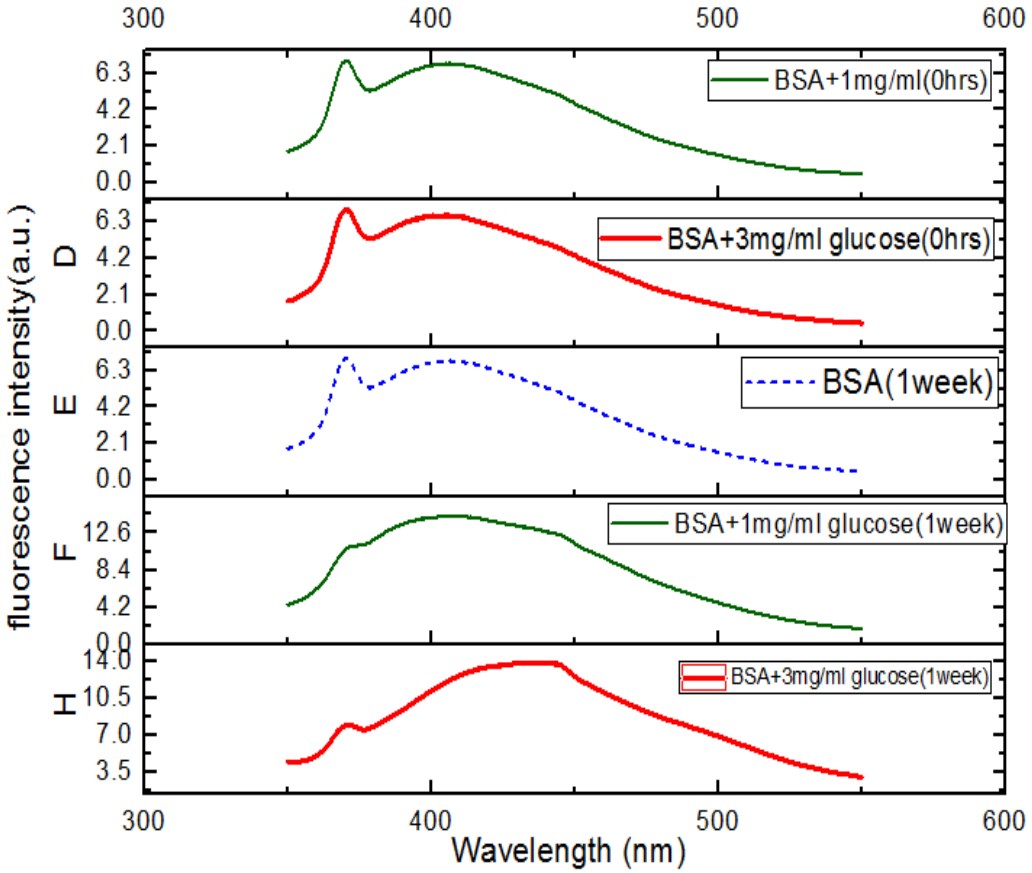

Figure 6- The formation of AGE indicated by the fluorescence peak at 440 nm in hyperglycemic condition.

After one week of incubation, the sample containing only BSA showed no emission at 440-450 nm range incompliance with freshly prepared BSA with different glucose concentration. The presence of the glucose induced structural changes in the protein as established by CD analysis showing increased beta strands (table4) with the increase in the

glucose concentration. The alteration in the structure of the protein due to interaction with glucose (at a concentration > 1mg/ml) after 1 week of incubation may expose hydrophobic residues of the protein increasing fluorescence. As the glucose forms hydrophobic interactions with the bovine serum albumin as shown by the docking studies (Figure 2), high concentration of the glucose leads to more such interactions thus exposing hydrophobic residues.

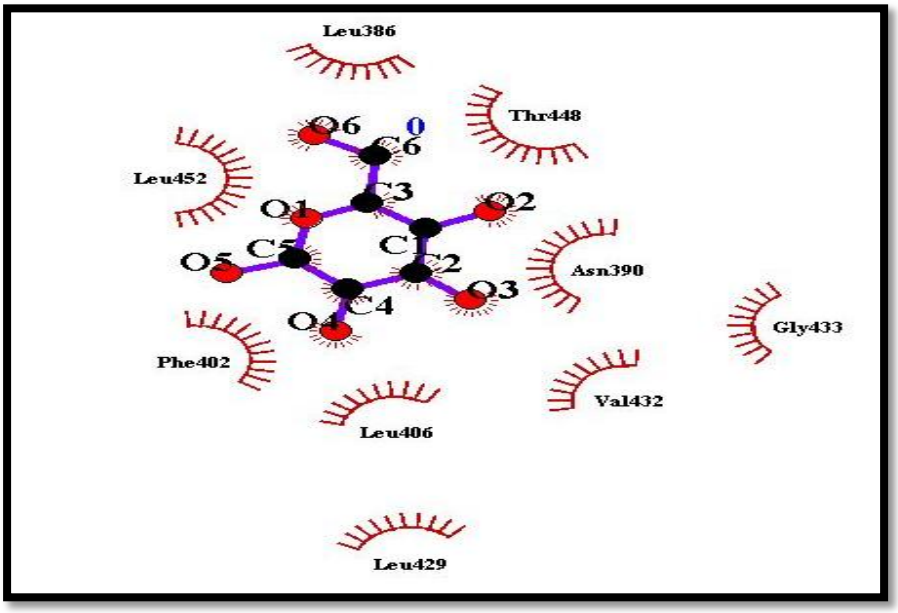

Figure 7- The plot showing hydrophobic interaction of the glucose with the residues of the BSA.

The structural changes due to these interactions caused the formation of the protein crosslink in the presence of the high concentration of the glucose resulting in the blue shift in the fluorescence peak towards 440nm. The fluorescent peak at 440 nm confirms the presence of pentosidine (21) and the crossline (the cross linked fluorescent AGEs) with BSA in hyperglycaemic condition.

4.2 Structural change in BSA due to AGE formation-

The serum albumin is the predominant protein present in the blood. Being the globular protein it has presence of the helices rather than beta strands as depicted in the ramachandran plot.

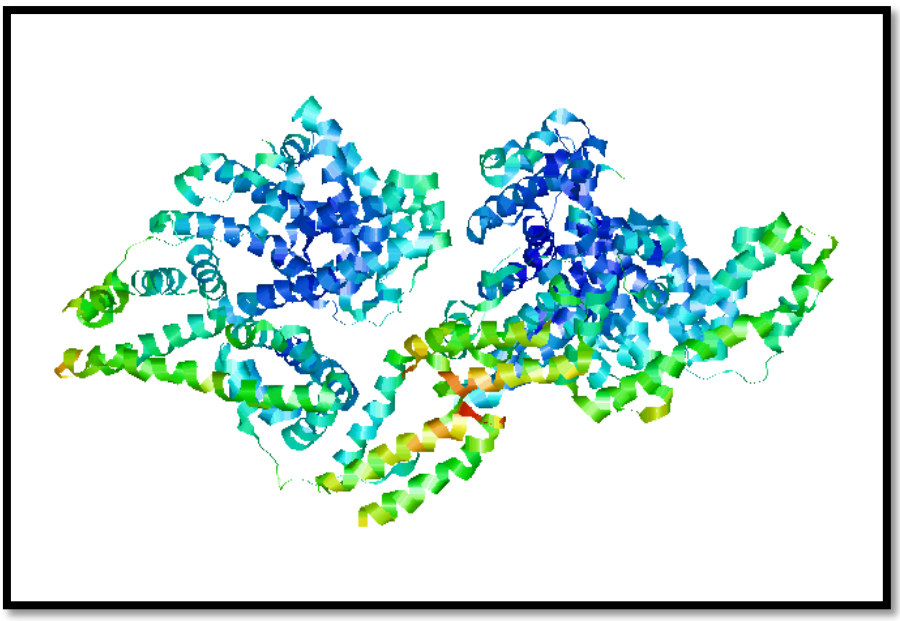

Figure 8- the 3D structure of the bovine serum albumin obtained from the protein data bank.

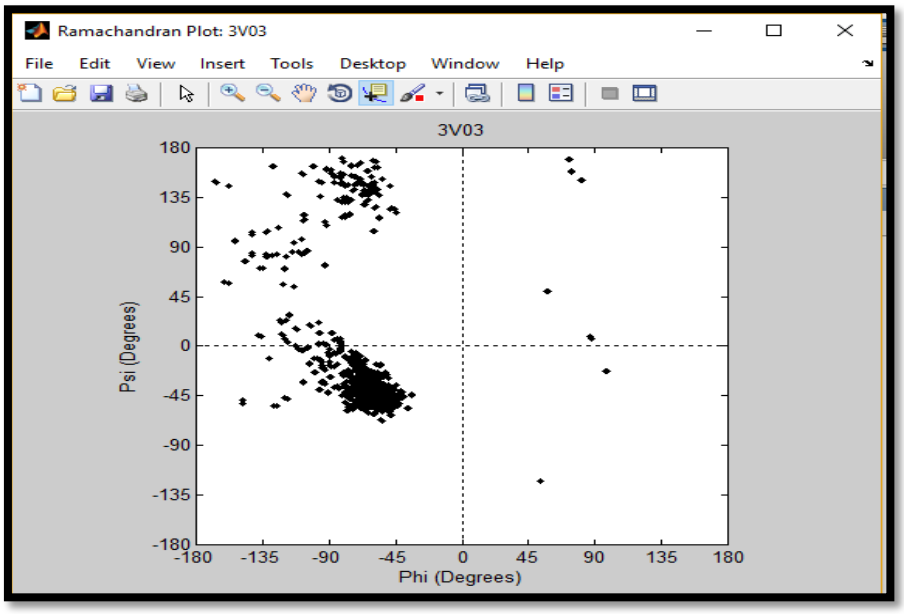

Figure 9- Ramachandran plot of the bovine serum albumin

The bovine serum albumin structure consists of 68 numbers of the helices and 67 turns but no beta strands as shown in the data depicted in Table 3 obtained from the Rasmol software.

Table 3- The types and number of the secondary structures present in the BSA

Types of secondary structure in the BSA	Number of secondary structures
helices	68
strands	0
turns	67

Thus in the native form the bovine serum albumin do not consists of the beta sheets as the secondary structures. Any change in the secondary structure present in the protein clearly alters the protein structure.

Table 4- The ratio of secondary structures present after AGE formation-

Ratio of secondary structures	BSA	BSA+1mg/ml glucose	BSA+3mg/ml glucose
Helix	37.2	38.2	33.3
Beta	8.5	4.3	21.2
Turn	22.1	24	17.6
Random	32.2	32.2	27.9
Total	100	100	100

In comparison with the CD analysis of BSA alone after one week of incubation in PBS, the BSA in the in 1mg/ml of the glucose concentration showed slight decrease in beta sheets compensated by increase in turns. Overall, the percentage of secondary structures present in the BSA measured after one week of incubation showed almost no variation in normal glucose concentration (1mg/ml). The increase in the concentration of the glucose to 3mg/ml increased the percentage of the beta strands to 33.3 compared to the control with just 8.5, pointing to the significant structural changes in the hyperglycaemic condition as shown in the table4.

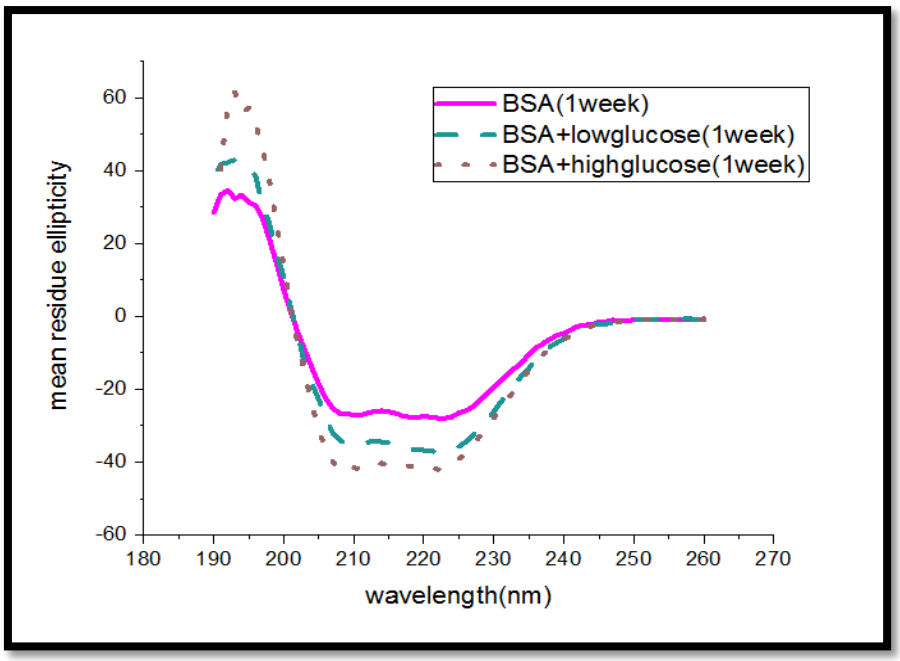

Figure 10- the secondary structures present in the BSA in different glucose concentration.

Previous studies (23) have already confirmed that the presence of higher percentage of beta strands is the structural characteristic of advanced glycation end products (AGEs).

4.3 The formation of the non-fluorescent AGEs in vitro-

The three types of AGEs has been described i.e. non fluorescent cross-linking AGEs, fluorescent cross linking AGEs and non –cross linking non-fluorescent AGEs. The fluorescence emission spectra at the 440-450 nm range indicated towards the formation of the fluorescent cross linking AGEs. The m/z ratio of the peptides obtained from the electron spray ionization mass-spectrometry (ESI-MS) for the one week-incubated BSA in the hyperglycaemic condition (Table 5) confirmed the formation of the non-cross linking non-fluorescent AGEs. One of the reversible intermediate for the formation of the AGEs by the amadori reagents is also detected at m/z ratio of the 553.64.

Table 5- the non fluorescent AGE detection with the m/z ratio of the peptide-

m/z ratio of peptide	Product formed
449.60	CML(carboxy methyl lysine)
553.64	Schiff base/AP
572.27	Pyrraline

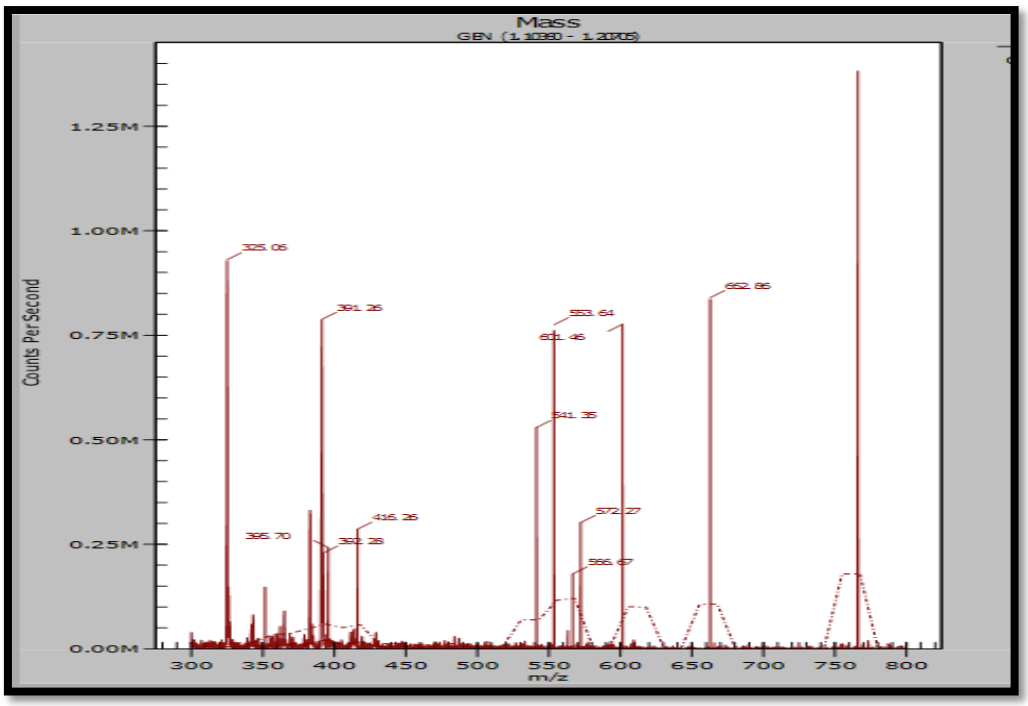

Figure 11- The m/z ratio of the 553.64 and 572.27 is the presence of the Schiff base and pyrraline in the *in vitro* glycation model with the BSA.

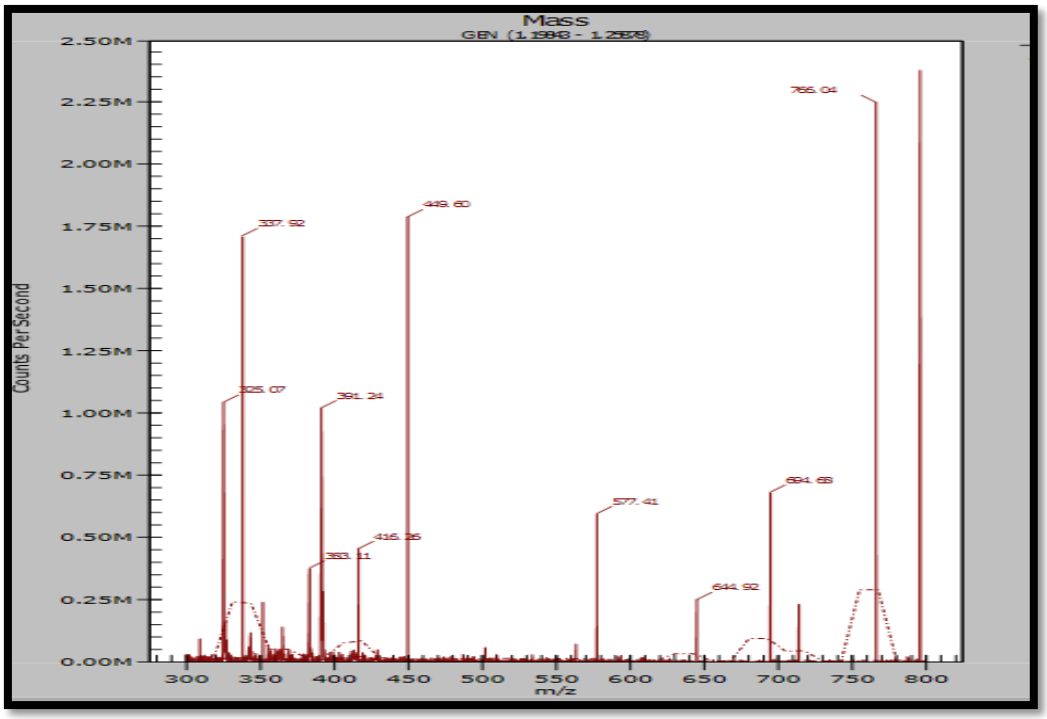

Figure 12- The peptide at m/z ratio of the 449.60 is the formation of the CML (carboxy methyl lysine).

The 449.60 m/z ratio of the peptide indicates the formation of the CML (carboxy methyl lysine), one of the early AGEs formed after glycation *in vivo*. The pyrraline, a non-

fluorescent AGE, was also formed as pointed by the m/z ratio of the 572.27 in the one week-incubated BSA under hyperglycaemic conditions.

The *in vitro* glycation model confirmed the formation of the both fluorescent and non fluorescent AGEs. The intensity of the 449.60 peak clearly indicates that one of the predominant AGEs formed is the CML (carboxy methyl lysine) among the non fluorescent non-cross linking AGEs.

4.4 The effect of inhibitors on the formation of the AGEs-

The AGEs lead to ROS generation, and the ROS generation accelerates gluco-oxidation which is one of the methods for the formation of the AGEs. To break this cycle of amplifying AGE formation, the antioxidant glutathione in the concentration of 2.5mg per ml was added. But contrary to predicted result, glutathione enhanced fluorescence intensity. This paradoxical result can be explained by the inability of in vitro environment to replenish reduced glutathione.

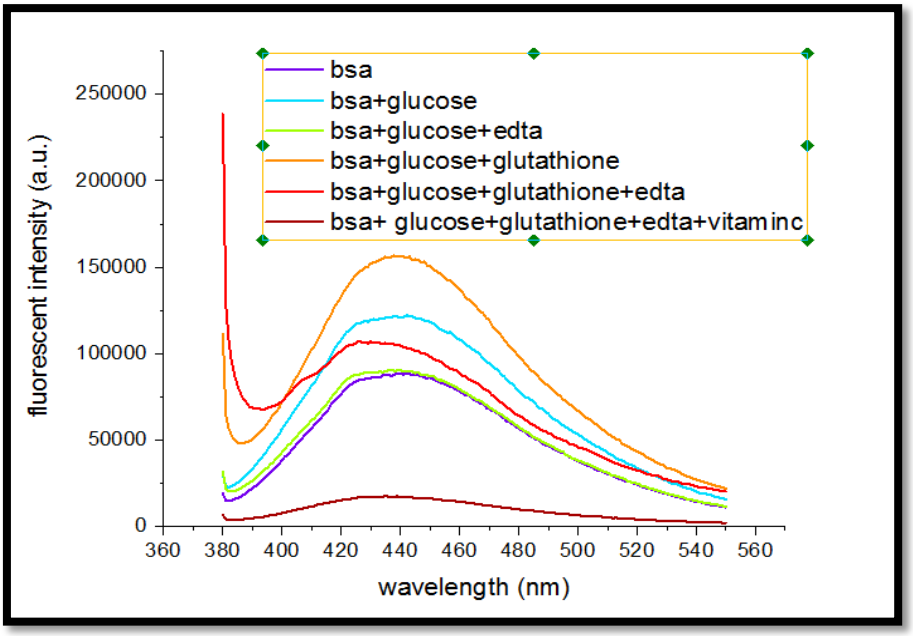

Figure 13- The relative fluorescent intensity at 440nm with addition of antioxidants and chelator.

Glutathione (GSH) donates electrons to reduce disulfide bridges to cysteines present in the protein and itself getting oxidized to glutathione disulfide (G-S-S-G). In the biological system, oxidized glutathione is reduced by NADPH so that the anti-oxidant property

continues. On the contrary, *in vitro* environment, the reduced glutathione was not replenished leading to a decrease in the GSH/G-S-S-G ratio.

This in turn, results in oxidative stress inducing structural changes in the protein and accelerating the formation of AGEs. Thus, ROS generation and protein glycation form a vicious cycle that initiates, accelerates and maintains the patho- physiological mechanisms leading to diabetic complications. From the docking study, the binding energy of the EDTA with the BSA was found to be -31.22 kcal/mol, lower than that of glucose with BSA (-35.40 kcal/mol). The preferential binding of the EDTA over glucose with BSA prevents binding of the glucose with subsequent reduction in glycation indicated by the fluorescent intensity close to fluorescence intensity of BSA alone. Since the binding residues on target glycated protein were found to be different, The EDTA probably acted as non-competitive inhibitor for the glucose to bind to BSA resulting in radical reduction in AGEs formation. At the same time, the combination of the glutathione, EDTA and ascorbic acid almost nullified the fluorescence at 440nm indicating that the binding of the EDTA stabilized the protein and the ascorbic acid antioxidant activity of ascorbic acid was complemented by the presence of the glutathione.

4.5 Macrophage activation in high glucose – NBT test-

The two main biological systems responsible for the ROS generation *in vivo* are the mitochondria and the NADPH oxidase (23).

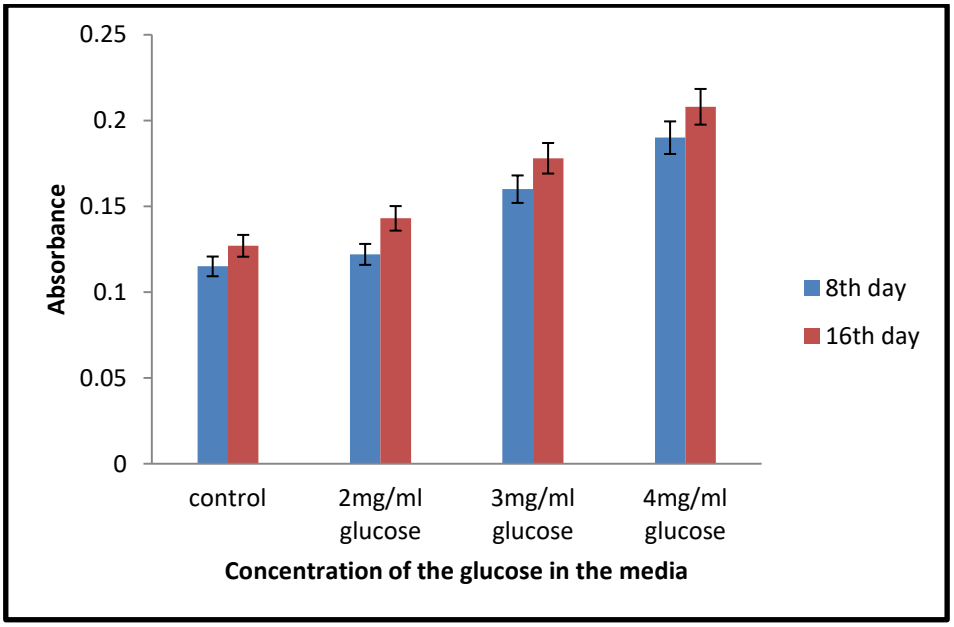

Figure 14- The macrophage activation for superoxide production in hyperglycemic condition.

The increase in glucose concentration increased the activity of NADPH oxidase and induced expression of iNOS causing more superoxide formation. Macrophages are highly active with increased superoxide production in high glucose (4mg/ml).The superoxide production enhanced with the increase in the duration of the exposure of the macrophages to the high glucose concentration. The unregulated glucose concentration in diabetic conditions enhances the ROS production leading to the diabetic complications.

4.6 Cell viability with the MTT assay-

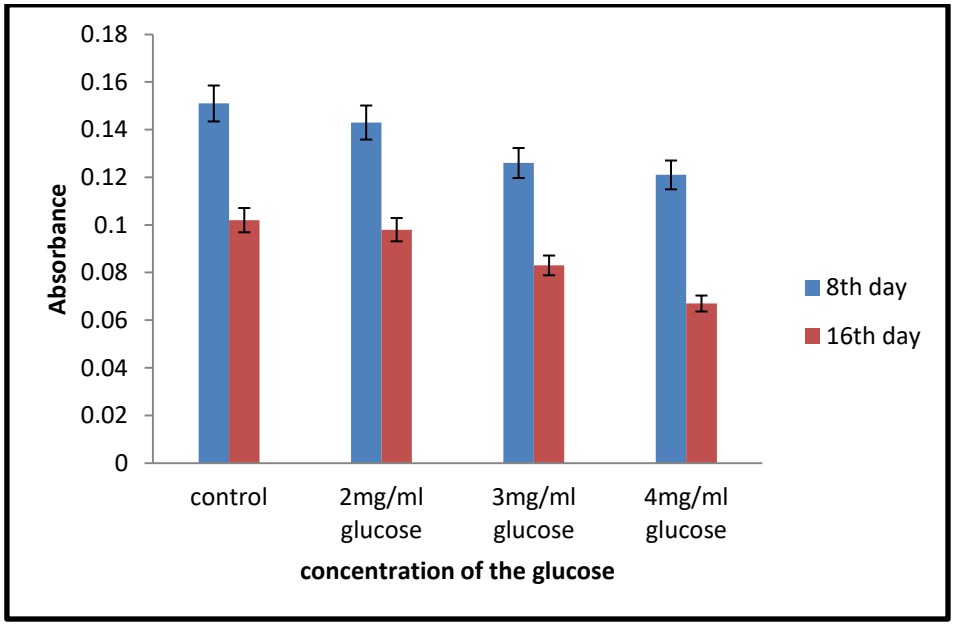

Figure 15- The MTT assay for the cell viability in high glucose.

As per the MTT assay results (Figure 15), the viability of the cells reduced with the increase in the time of exposure to the high glucose environment. The diminished cell viability in response to high glucose was further verified by trypan blue test. The high glucose induced ROS generation due to dysfunctional mitochondria validates the impaired activity of the dehydrogenase complex of the mitochondria responsible for the high ROS production.

The MTT determines the metabolic state of the cell. The dehydrogenase of the mitochondrial enzyme complex reduces MTT to the formazan during normal metabolic activity of the cell representing cell viability. Under the influence of the high glucose concentration the dysfunctional mitochondria accelerates ROS generation. In the normal metabolic state, only complex 3 of the mitochondria is involved in the ROS generation. In the non haemostatic condition the complex 1, 2 and complex 3 enhances ROS production as a consequence of the mitochondrial dysfunction. The activity of the complex 1 and complex 2 is determined by the

MTT assay. The high glucose condition augments ROS generation above physiological limit that can lead to immediate cell death or enhance AGE formation.

4.7 Types of ROS generated-

The H2DCFDA dye detects the formation of overall oxygen free radicals (hydrogen peroxide, cytosolic super oxides, mitochondrial super oxides, hydroxy radicals etc).

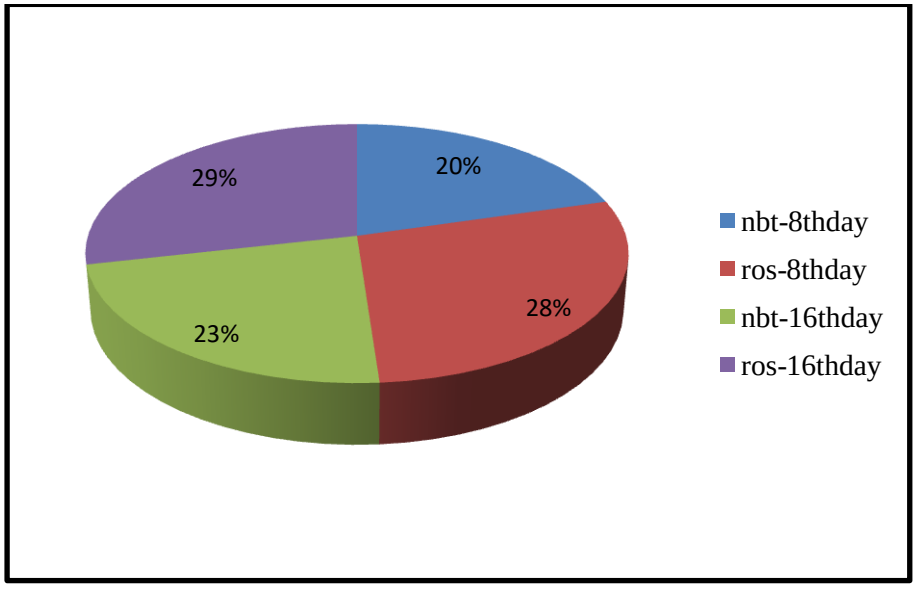

Figure 16- The graph indicates increase in intracellular ROS generation with time under hyperglycemic condition

A substantial increase in all types of intracellular ROS was found with increasing glucose concentration from 2mg/ml to 4mg/ml. The results complemented the results obtained from NBT test. Thus high concentration of intracellular ROS being produced due to hyper activity of enzyme systems involved in superoxide production such as nitric oxide synthase and NADPH oxidase and mitochondrial superoxide production. The dye detects the formation of the both the intracellular and the extracellular reactive oxygen species produced by the mitochondria and the NADPH oxidase. The superoxides are the one of the predominant ROS generated in vivo.

The NBT test only detects the superoxide production by the NADPH oxidase. Thus as indicated in the pie chart the predominant ROS generated is the superoxide free radicals among all the free radicals produced. The high glucose concentration increased the production of ROS with increase in incubation time. Thus all type of ROS generation pathways are hyper active under the influence of high glucose.

4.8 Intracellular ROS vs. Extracellular ROS-

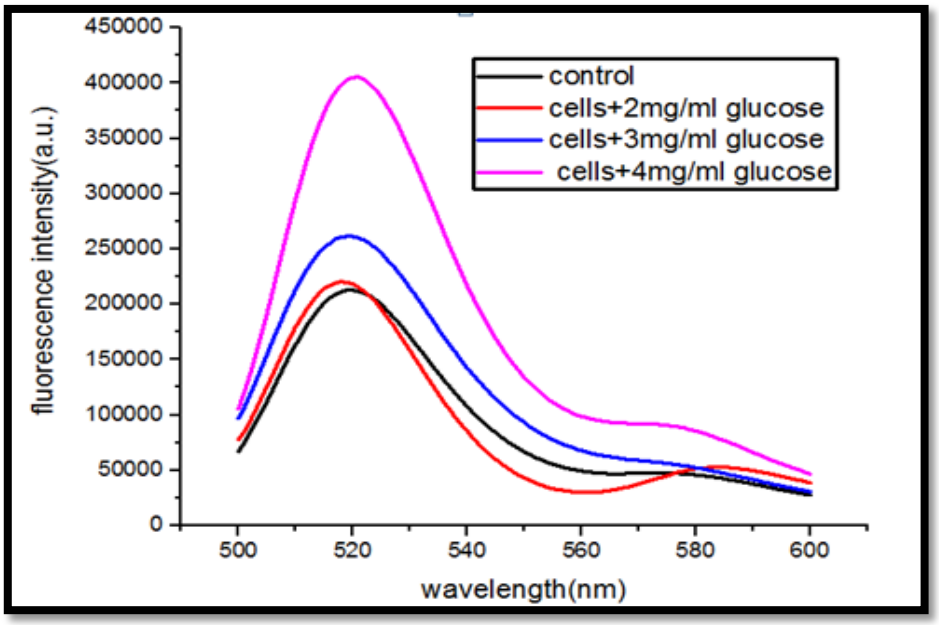

Figure 17- the graph representing greater intracellular ROS generation than extracellular in macrophages

The high intracellular and extracellular ROS production is the main culprit behind inflammation, cell death.

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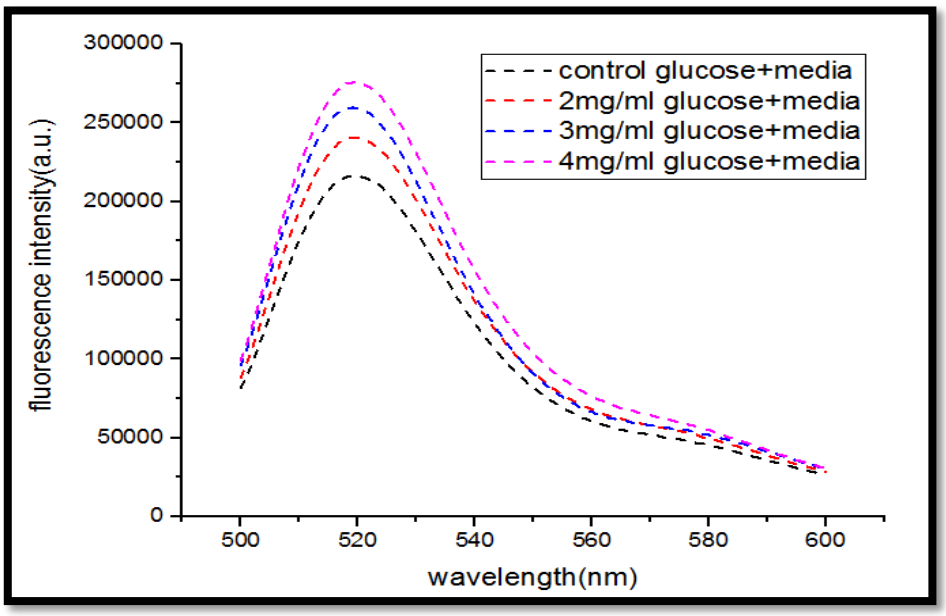

Figure 18- the less extracellular ROS produced in macrophages in hyperglycemic condition.

The mitochondrion is the main source for the free radical production inside the cell causing protein oxidation, lipid peroxidation and other metabolic alterations. The enzyme system NADPH oxidase present on the plasma membrane of the phagocytic cells releases both the extracellular and intracellular superoxides. The hyper activation of both the systems by various ligand and receptor interaction generates elevated levels of the ROS altering the structure and function of the biomolecules present inside the cell and the cell matrix.

The graph points to that with the increase in the glucose concentration; the intracellular ROS production is also elevated by the macrophages. The highest fluorescent intensity obtained in the cells incubated with the 4mg/ml of the glucose in the media with respect to the control.

Only few membrane bound enzyme systems are involved in the extracellular ROS generation such as NADPH oxidase. The increase in the glucose concentration in the media used for the culturing macrophages enhanced the formation of the extracellular ROS. The more intracellular ROS generated in comparison to the extracellular ROS shows that the mitochondria and various other systems are involved in the intracellular ROS production. The mitochondrion is the predominant source for the generation of the ROS.

4.7 Comparing mitochondrial activity with the ROS generation-

The high glucose inside the cell causes more glucose to get oxidized in the TCA cycle, pushing more electron donors (NADH and $FADH_2$) into the electron transport chain.

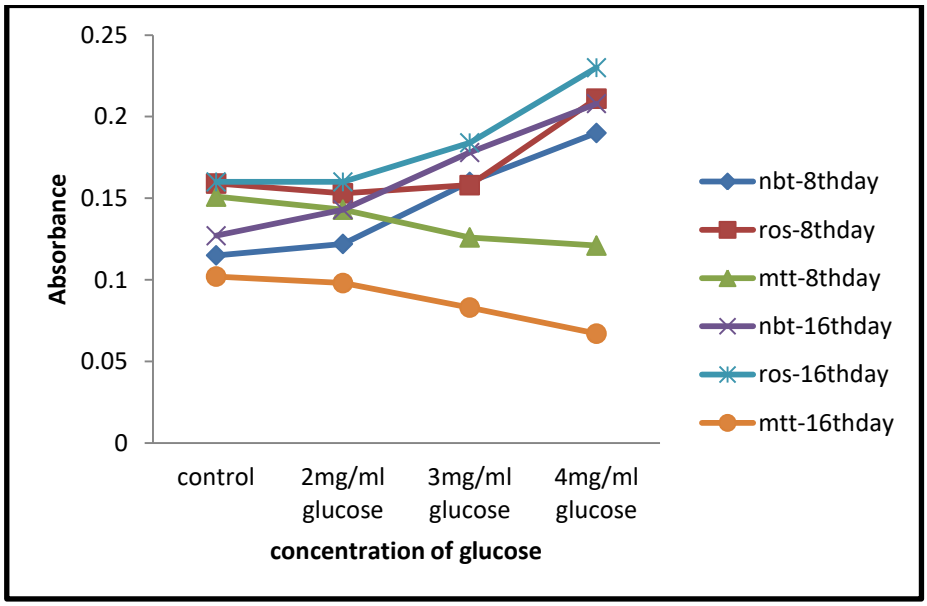

Figure 19- The graph representing comparative analysis of role of mitochondria in ROS generation.

As a result the voltage gradient across the mitochondrial membrane increases until a critical threshold is reached. At this point electron transfer inside complex 3 is blocked, causing the electrons to back up to co-enzyme Q, which donates the electrons one at a time to molecular oxygen, thereby generating superoxide.

Based on in vitro studies it has been hypothesized that excess production of O_2^- is via the premature collapse of the mitochondrial membrane potential so that electrons leakage from the complex1 to form superoxide and hydrogen peroxide rather than ATP. In high glucose at 8^{th} and 16^{th} day significant collapse of the mitochondrial membrane potential observed with increase in ROS generation by the cell.

4.8 The glycation of the human serum albumin –

The formation of the in vitro AGEs has been established using BSA as in vitro model.

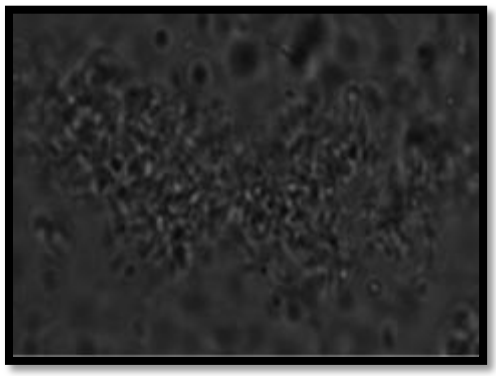

Figure 20- Human Serum albumin (HSA) showing no fluorescence in the emission range of 440-470nm

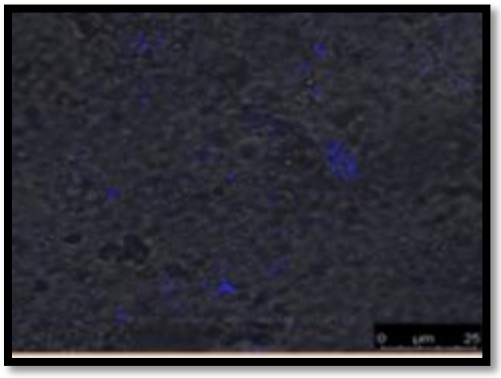

Figure 21- HSA incubated with the glucose for one week showing fluorescence in the emission range of 440-470nm

Based on the results obtained with the BSA incubated in hyperglycemic conditions for the one week forming both fluorescent and the non-fluorescent AGEs , the human serum albumin incubated with the 3mg/ml of the glucose after one week was added to the media of the

macrophages. The blue emission in the range of the 440-470 nm was observed under confocal microscope. The human serum albumin in the absence of the glucose shows no fluorescence as shown in the figure 20. The addition of 3mg/ml of the glucose increased the fluorescence at 440nm (the blue fluorescence) as depicted in the figure 21.

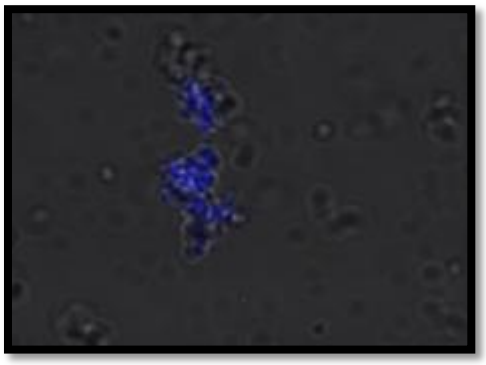

Figure 22- HSA incubated with the glucose for 2weeks showing increased fluorescence in the emission range of 440-470nm

The increase in the fluorescence with the increase in the incubation time was observed as shown in the figure22. The formation of the fluorescent AGEs has been established with the human serum albumin in the hyperglycaemic conditions.

4.9 The formation of the intracellular AGEs in the macrophages-

There is formation of the both intracellular and extracellular AGEs in the influence of the high glucose concentration. The amadori rearrangements and the auto-oxidation are the main mechanisms involved in the formation of the AGEs. In the cells both the pathways are active as the high influx of the glucose through glycolytic and polyol pathways forms intermediates such as glyceraldehydes-3-phosphate , dihydroxyacetone phosphate generating alpha dicarbonyl precursors involved in the formation of the advanced glycation end products.

Figure 23- The macrophages after one month of the culturing in low glucose media showed no formation of the intracellular AGEs.

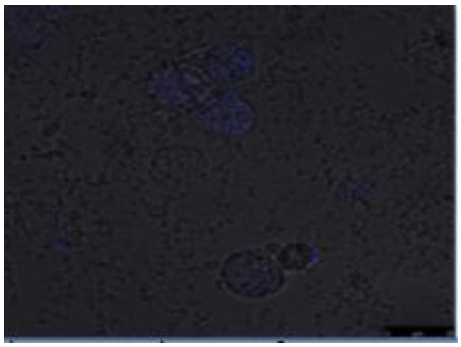

Figure 24- The macrophages after one month of the culturing in high glucose showed intracellular AGEs formation.

The auto-oxidation of the glucose in the presence of the reactive oxygen species formed inside the cells under high glucose condition forms alpha dicarbonyl precursor as a consequence intracellular AGEs formation is being contributed by both the mechanisms.

4.10 The interaction of the AGEs with the receptors present on the surface of macrophages-

The macrophages express RAGE and scavenger receptor on their surface.

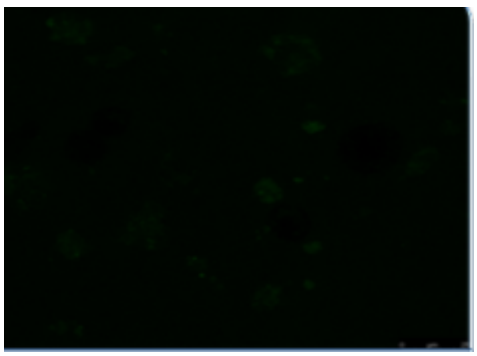

Figure 25- The macrophages cultured for 1 month in 2mg/ml of the glucose concentration show very less ROS generation.

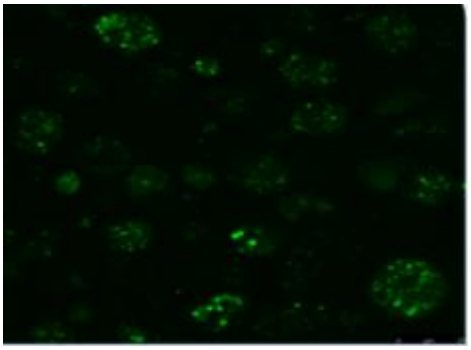

Figure 26- The increased ROS production due to addition of the extracellular AGEs in the media of the macrophages.

The interaction of the formed AGEs with these receptors activates NADPH oxidase, increases expression of the NFκB, PKC isoforms and mitochondrial reactive oxygen generation.

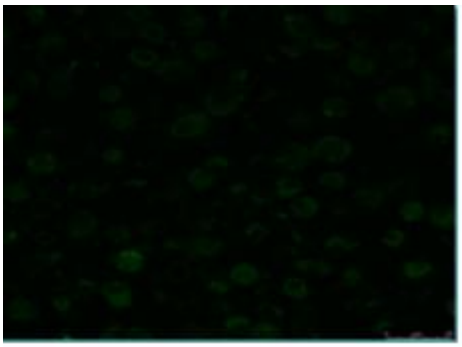

Figure 27- The increase in the fluorescence intensity at 523 nm with 3mg/ml of the glucose in the media

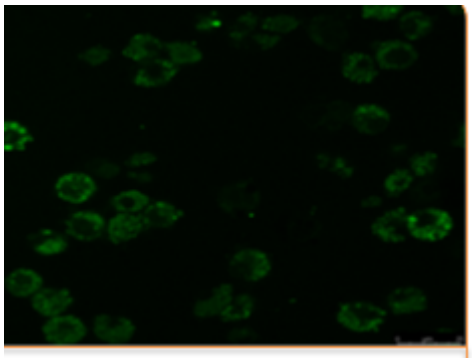

Figure 28- The addition of the extracellular AGEs elevated ROS production compared to the 2mg/ml.

The AGEs formed in the in vitro glycated human serum albumin with the 2mg/ml, 3mg/ml and 4mg/ml of the glucose concentration respectively was added to the macrophages growth media containing 2mg/ml, 3mg/ml and 4mg/ml of the glucose respectively. After one week of the incubation at 37ºc the samples were analyzed for the ROS generation. The macrophages are growing in the high glucose concentration from one month which definitely increased the expression of the RAGE on the surface of the macrophages.

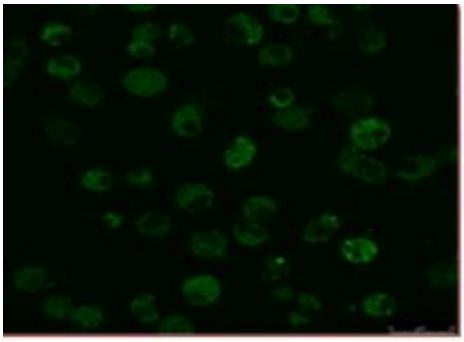

Figure 29- The fluorescence increase at 523nm in 8mg/ml of the glucose

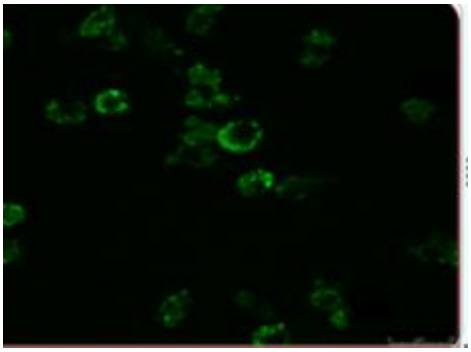

Figure 30- High ROS generation in the high glucose concentration of the 8mg/ml

Thus extracellular AGEs added to the media interacted with these receptors, further increasing their expression and with the CD36 scavenger receptor activating ROS generating targets NADPH oxidase and the mitochondria.

4.11 Effect of AGEs interaction with the receptors on oxidative stress and eveluating glutathione anti-oxidant property for the ROS generated in high glucose condition –

The added AGEs in the media of the macrophages enhanced the ROS generation within 24hrs of incubation as indicated by the increase in the fluorescent intensity at 523nm using H_2CMDCFDA dye.

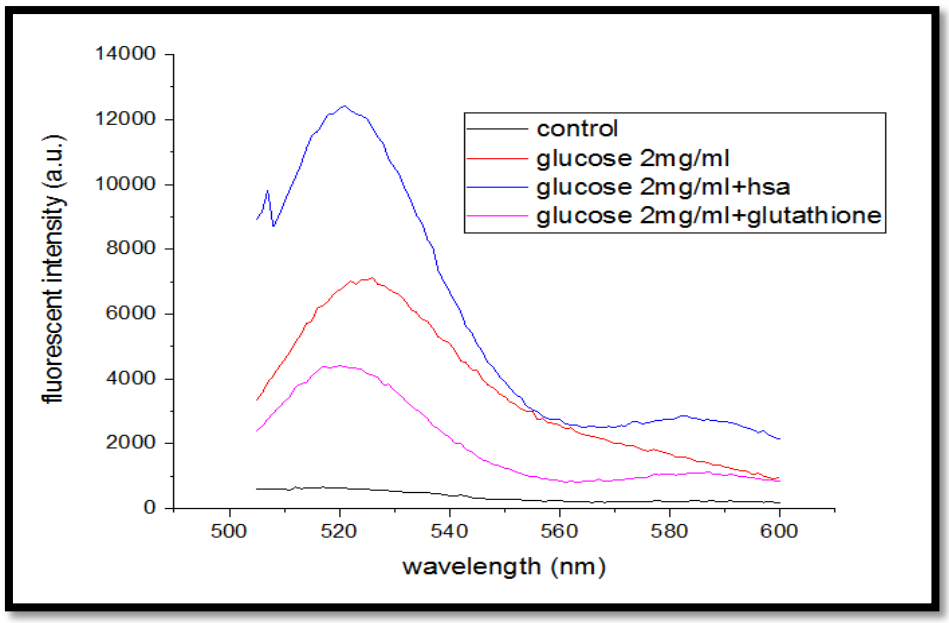

Figure 31- The glutathione inhibits ROS generation.

Glutathione acts as scavenger for the reactive oxygen species generated in the human cells. The reduced form of the glutathione(GSH) gets oxidized to (G-S-S-G) in the process. The 2.5 mg of the glutathione was added and incubated for 24hrs. The reduced fluorescent intensity confirm that the GSH reacted with the extracellular ROS therefor decreasing the fluorescent intensity.

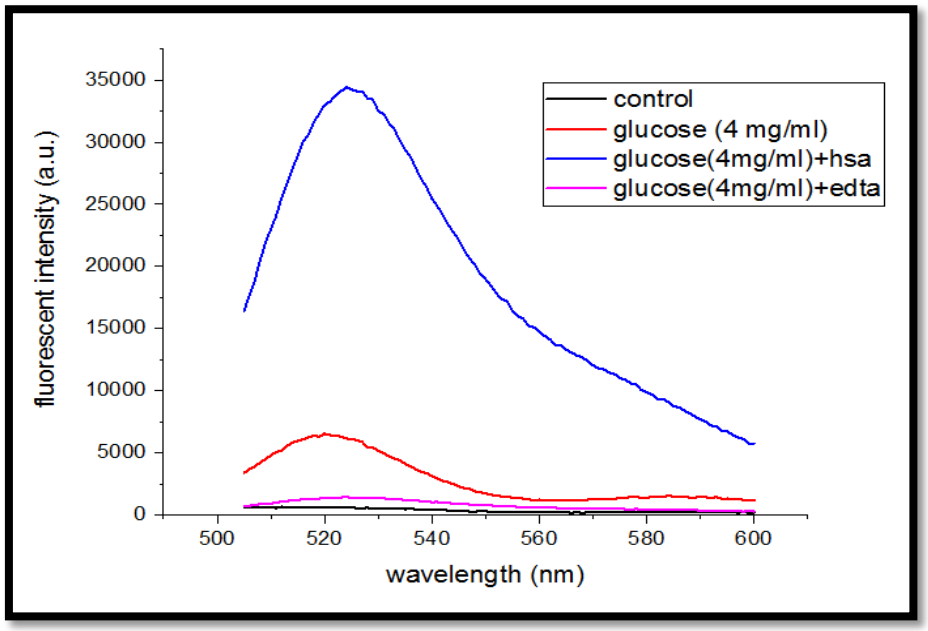

Figure 32- the fluorescent intensity at 523 nm to check ROS generation with HSA and EDTA.

The reduced fluorescent intensity by addition of the EDTA decreased the overall ROS generated by the macrophages within 24hrs of the incubation. EDTA has preferential binding for both the proteins and the glycated proteins compared to the glucose. The binding of the EDTA with the protein helps protein to revert to the native form, thus high chances of the bound glucose being release and also preventing further binding with the glucose. The reduced intracellular AGEs concentration is having direct co-relation with the reduced ROS generation by the cells.

The high glucose induced increased ROS production as confirmed by the NBT test. The greater ROS formation and also the high glucose conditions further lead to the formation of the intracellular AGEs by both the process of auto-oxidation of the glucose and the amadori rearrangements respectively. The intracellular AGEs formed and the interaction of the extracellular AGEs with the cells further exuberated ROS generation.

4.13 Determining cyto-toxicity of the EDTA with the MTT assay-

The 1.5mg/ml of the concentration used for the inhibition of the AGE formation is not cytotoxic to the cells. The viability of the cells is getting decreased with the addition of the extracellular AGEs to the macrophages. The higher the amount of the glycation and more the elevated glucose level causing significant decrease in the viability of the cells. As shown in the figure33.

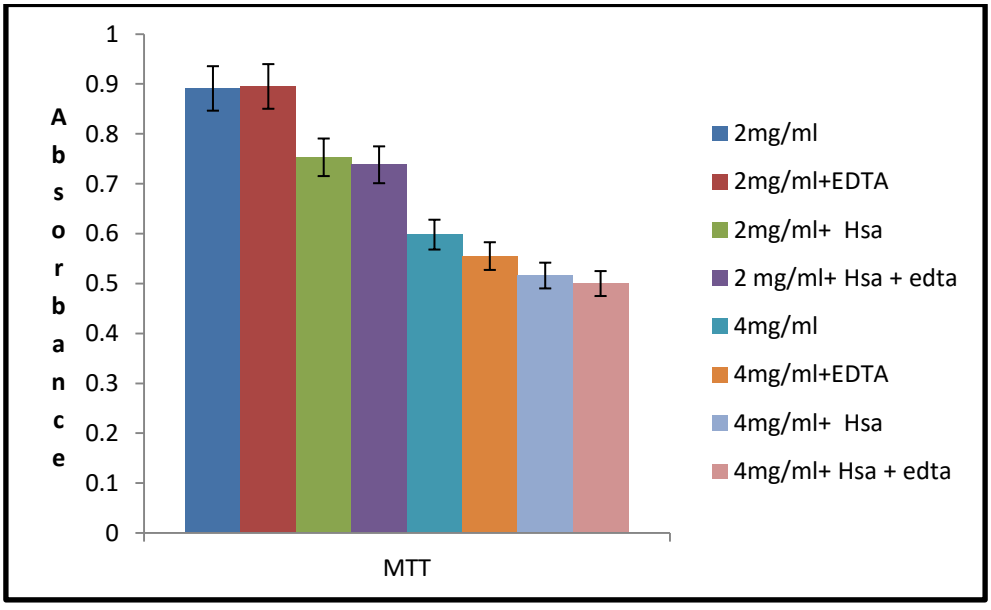

Figure 33- MTT assay to determine cyto toxicity of the EDTA.

The decrease in the viability is the indication towards the mitochondrial loss of the mitochondrial membrane potential.

4.14 The interaction of the AGEs with the receptor present on the surface of the macrophages and role of EDTA –

The interaction of the AGEs with the receptors present on the surface of the macrophages (RAGE and scavenger receptors) is significantly enhancing the ROS generation in the cells as compared to the high glucose induced cells.

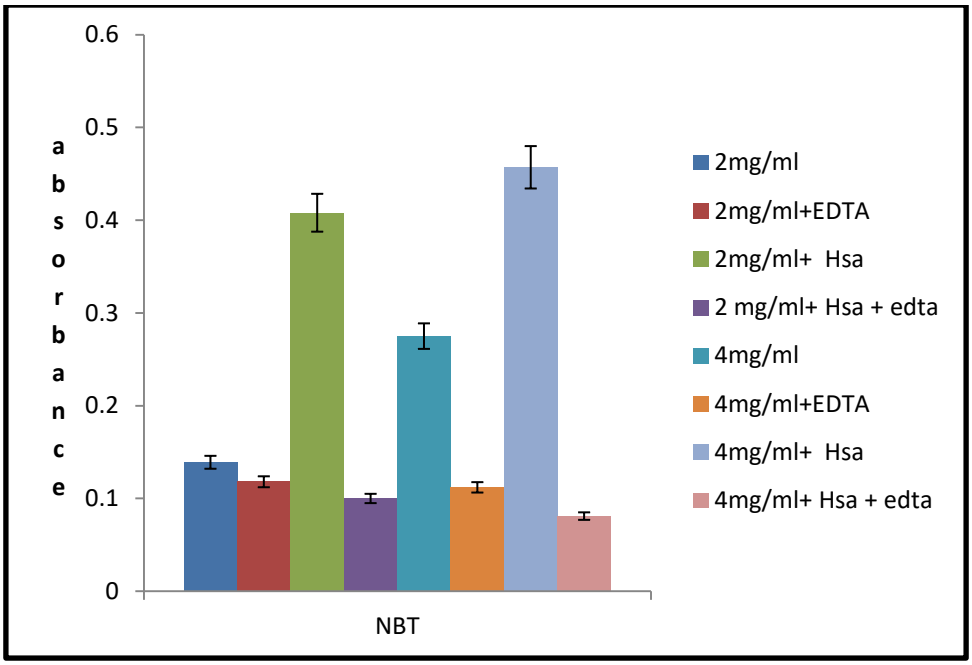

Figure 34- NBT assay for the ROS generation

The addition of the EDTA is inhibiting both the intracellular and the extracellular formed AGEs. The reduction in the amount of the AGEs present is having direct consequences on the decreased ROS generation by the NADPH oxidase.

The 1.5 mg/ml of the EDTA added is significantly reducing both the intracellular and the extracellular superoxides generated by the NADPH oxidase. The same concentration of the EDTA is effective against the high concentration of the AGEs formed in the case of the 4mg/ml glucose used for the culturing of the macrophages. Thus the deprivation in the AGEs is definitely linked with the reduction in the reactive oxygen species.

4.15 Comparisons of the MTT and NBT results with EDTA as inhibitor-

The concentration of the EDTA used for the inhibitory studies is not cyto- toxic to the cells. The 1.5mg of the EDTA used is effective in inhibiting the intracellular AGEs formed due to

the culturing of the macrophages in the high glucose condition for one month. The same concentration is also effective for the inhibition of the extracellular AGEs added to the macrophage media therefore preventing the interaction of the extracellular AGEs with the receptors present on the macrophages.

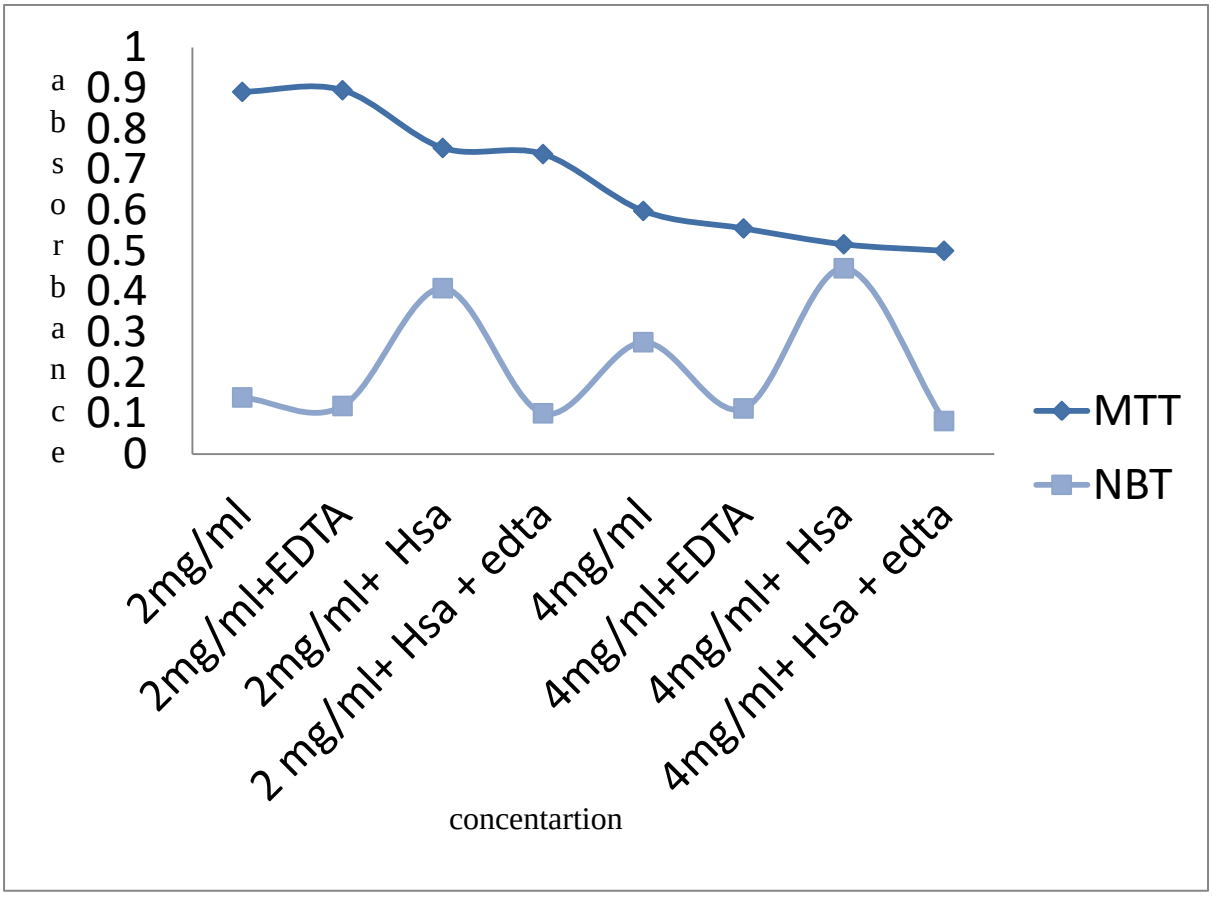

Figure 35- The comparative study of MTT and NBT assay

The viability of the cells further decreased because of the generation of the more ROS due to the extracellular AGEs. The higher the amount of the formed AGEs the more is the activation of the pathways involved with the reactive oxygen species formation. The diabetic patients with highly unregulated glucose concentration are at high risk of the early diabetic complications.

4.16 AGE formation in diabetic serum and inhibitory studies-

The serum collected from 5 diabetic patients showed AGE formation with varied fluorescent intensities.

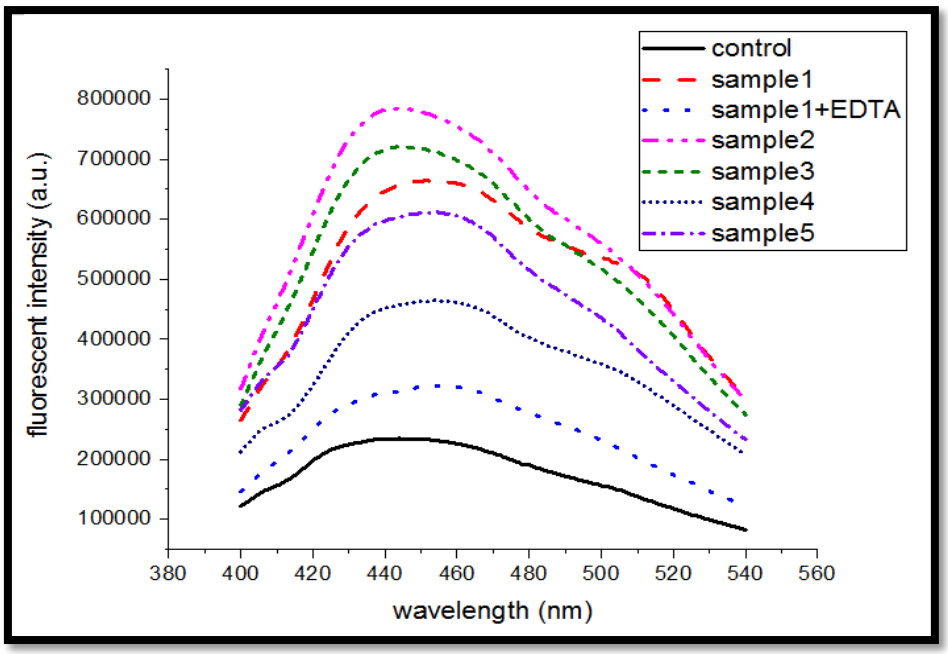

Figure 36- The diabetic serum based studies for the AGE formation.

The highest fluorescent intensity observed in serum of sample2 having glucose concentration of 191mg/ml.The lowest fluorescent intensity observed in the sample4 serum with glucose concentration of 153mg/ml.The 1.5mg EDTA successfully inhibited AGE formation in the sample1 as indicated by decrease in fluorescent intensity as compared to fluorescent intensity in serum without inhibitor. With respect to the control serum (non-diabetic serum) having 94mg/ml glucose concentration in the isolated serum the diabetic patient serum has high concentration of the AGEs present in the serum. The high the concentration of the circulating AGEs in the blood the more the patient is prone to renal failure and arthrosclerosis due to accumulation of these AGEs in the glomaerulus filtration unit of the nephron and in the blood vessels respectively. In the endothelial and macrophages these extracellular AGEs interact with the receptors present on the surface of these cells causing increase in the formation of the inflammatory cytokines further enhancing susceptibility to diabetic related complications. The intracellular AGEs cause lipid peroxidation, protein oxidation and changes in DNA structure. The structural and the functional changes in the biomolecules lead to the cell death.

4.17 The role of the ascorbic acid and EDTA against the AGEs formed in the diabetic serum-

The 2.5/ml mg of the ascorbic acid used as anti-oxidant increased the formation of the AGEs in the serum instead of inhibiting. The pro-oxidant activity of the ascorbic acid and also involvement of the SN2 nucleophilic addition reactions of the ascorbic acid with the proteins are the main reasons accelerating formation of the AGEs.

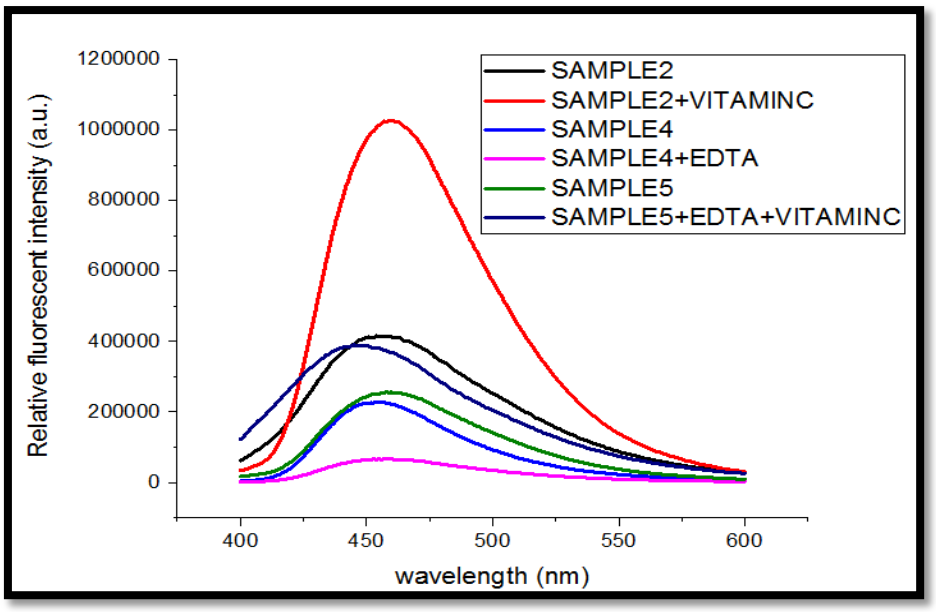

Figure 37- Inhibitory studies with the diabetic serum

The pro-oxidant activity of the ascorbic acid is due to involvement in the fenton reaction generating hydroxy free radicals. The hydroxy free radicals further causing glucose auto-oxidation thus AGE formation in the serum with the ascorbic acid.

The addition of the ascorbic acid with the EDTA showed less increase in the fluorescent intensity as compared to the ascorbic acid alone in case of the sample 2 depicting 50% increase in the fluorescent intensity as shown in figure 37. The EDTA is definitely preventing amadori rearrangements and also chelating the transition metal present in the serum therefore inhibiting AGE formation. The auto-oxidation pathway is still active for the AGE formation due to pro-oxidant activity of the ascorbic acid thus there is net increase in the fluorescent intensity. The addition of the only EDTA is sufficient to inhibit the progression of the AGE formation in the serum. The EDTA is the effective inhibitor for the AGEs both in the in vitro model and also in the macrophages.

Chapter 5

5.1 Summary

The in vitro glycation model using bovine serum albumin (BSA) established the formation of the both fluorescent and non-fluorescent advanced glycation end products (AGEs) in the hyperglycaemic condition. The amount of AGEs formed increased with duration of the exposure to the hyperglycaemic condition. CD study confirmed the increase in beta sheets in secondary structure of glycated BSA that might prompt further progression AGEs formation.

The high glucose triggered the ROS generation from mitochondria and the NADPH oxidase as verified from NBT and H2DCFDA assays. The cell under oxidative stress accelerated intracellular AGE formation via auto-oxidation pathway (20). The study concluded that protein glycation and ROS generation establishes a vicious cycle inside the macrophages that lead to progression in diabetic complications.

The isolated diabetic serum showed substantial increase in the presence of the AGEs as compared to the control. These results are in compliance with the *in vitro* glycation model. The supplement of the ascorbic acid to the diabetic serum augmented AGE formation due to its pro-oxidant activity. This can be explained by formation of Schiff base with the proteins in the presence of ascorbic acid that further accelerates AGE formation by amadori rearrangements (38).

5.2 Conclusion

The current study made an in depth patho- physiological investigation of protein glycation both in vitro and in situ. The results confirmed that protein glycation and ROS generation complement to each other and establish a vicious cycle in diabetic patients. Thus, by targeting either pathway can pave the way to a novel therapeutic solution for diabetic complications as whole.

5.3Future scope

- The metacentric study involving more diabetic patients needed for further exploration.
- The experimental study would be integrated effectively with in silico study to narrow down the process of pathway selection that leads to protein glycation and ROS generation.

- Clinical trial on use of ascorbic acid alone or in combination with glutathione and other anti-oxidants is needed to clarify the role of vitamin c in diabetic complications.

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